

Therapeutic dendritic cell vaccination against human papillomavirus

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Till min familj

ABSTRACT

Cervical cancer, which is caused by infection with human papillomavirus (HPV), is the second most common cancer among women worldwide, leading to 230000 deaths annually. The two currently available HPV vaccines can only be used prophylactically, so they are of no use to the 291 million women who are already infected and at risk of developing cancer. Early stage cervical cancer can be treated with surgery, chemotherapy, and radiation, although the treatment outcomes for patients with recurrent disease are poor. The main goals of this thesis were to develop and evaluate a possible treatment against HPV-induced cervical disease.

HPV uses several different mechanisms to evade elimination by the immune system, e.g., suppression of inflammation in the infected epithelium and down-modulation of the presentation of HPV antigens on MHC molecules, thereby avoiding recognition by immune cells. The use of TLR ligands as local treatments to overcome HPV-induced down-modulation of immune responses has been investigated. The results show that intravaginal or intratumoural administration of TLR ligands enhances the expression of both MHC molecules and chemokines, and promotes the influx of immune cells into the targeted tissues.

Dendritic cells (DCs) sense danger and activate antigen-specific T cells in the adaptive immune system, leading to the killing of virus-infected cells. The use of DCs as a therapeutic cancer vaccine was assessed in mice with tumours that expressed the HPV16 E7 antigen. The antigen used, E7, was chemically conjugated to cholera toxin (CT-E7), to enhance both antigen uptake and presentation and DC maturation. Vaccination with CT-E7-DCs primed for tumour-specific cytotoxic T cells led to a significantly reduced tumour burden. Complete tumour eradication was achieved by combining CT-E7-DC vaccination with intratumoural administration of a TLR ligand. The CT-E7-DCs also activated E7-specific human T cells, as demonstrated *in vitro* using blood cells from patients with HPV-induced cervical disease. The CT-E7-pulsed DCs produced IL-12 and induced E7-specific CD4⁺ T-cell responses, including the production of IFN- γ , which is crucial for a robust anti-tumour response. Indoleamine 2,3-dioxygenase (IDO), which is an enzyme produced by DCs, is proposed to affect negatively the outcome of DC vaccination owing to its down-regulation of T-cell responses. The results presented in this thesis show that exposure of DCs to CT does not induce IDO transcription or activity, whereas it does prime DCs for CD40 ligand-induced IDO production.

The results outlined in this thesis highlight the potential of a combinational immunotherapeutic treatment against HPV-induced cervical disease, indicating that it may be possible in the future to treat patients who have HPV-induced malignancies.

Keywords: human papillomavirus, therapeutic dendritic cell vaccination, cholera toxin, T cells, TLRs and CpG

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

- I **Nurkkala M**, Nordström I, Telemo E, Eriksson K.
MHC expression and chemokine production in the murine vagina following intra-vaginal administration of ligands to toll-like receptors 3, 7 and 9.
Journal of Reproductive Immunology 2007 Apr; 73(2):148-57

- II Chandy AG, **Nurkkala M**, Josefsson A, Eriksson K.
Therapeutic dendritic cell vaccination with Ag coupled to cholera toxin in combination with intratumoural CpG injection leads to complete tumour eradication in mice bearing HPV 16 expressing tumours.
Vaccine 2007 Aug; 25(32):5037-46

- III **Nurkkala-Karlsson M**, Wassén L, Nordström I, Gustavsson I, Slavica L, Josefsson A, Eriksson K.
Conjugation of HPV16 E7 to cholera toxin enhances the HPV-specific T-cell recall responses to pulsed dendritic cells *in vitro* in women with cervical dysplasia.
Submitted for publication

- IV **Nurkkala-Karlsson M**, Slavica L, Karlson T, Ingelsten M, Eriksson K.
IDO expression and functional activity in dendritic cells exposed to cholera toxin.
Manuscript

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ABBREVIATIONS

APC	Antigen-presenting cell
cDC	Conventional dendritic cell
CD	Cluster of differentiation
CIN	Cervical intraepithelial neoplasia
CpG ODN	Cytidine-phosphate-guanosine oligodeoxynucleotide
CT	Cholera toxin
CTB	Cholera toxin B subunit
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
E1-2, 4-7	Early proteins 1-2, 4-7
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte/Macrophage-Colony Stimulating Factor
HLA	Human leukocyte antigens
HPC	Haematopoietic progenitor cell
HPV	Human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
IDO	Indoleamine 2,3-dioxygenase
IFN- γ	Interferon- γ
IL	Interleukin
IP-10	Interferon gamma-inducible protein 10
IRF	Interferon regulatory factor
i.v.	Intravenous
L1 and L2	Late proteins 1 and 2
LSIL	Low-grade squamous intraepithelial lesion
MHC	Major histocompatibility complex
MIP-1 α	Macrophage inflammatory protein 1 α
moDC	Monocyte-derived dendritic cell
NF- κ B	Nuclear factor κ B
NK cell	Natural killer cell
PAMP	Pathogen-associated molecular pattern
Pap smear	Papanicolaou smear
pRb	Retinoblastoma protein
PRR	Pathogen-recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
s.c.	Subcutaneous
TAP	Transporter associated with antigen processing
Th1	T helper 1
Th2	T helper 2
TLR	Toll like receptor
Treg	Regulatory T cell

INTRODUCTION

Overview of the immune system

The human immune system, which comprises a constellation of responses that protects the body from microbial attack and from cancer, is divided in the innate immune system and the adaptive immune system. The innate immune system provides immediate host defence against pathogens, and includes the physical barriers that protect the body (e.g., the skin and mucosa, as well as chemical barriers, such as saliva, tears, and secretions), innate cells (leukocytes, such as neutrophils, monocytes/macrophages, natural killer cells, and dendritic cells), and soluble factors (e.g., the complement system, pro-inflammatory cytokines, and acute phase proteins) [1]. The adaptive immune system consists of the antigen-specific immune responses performed by the T cells and B cells, and their respective products, i.e., cytokines and cytotoxic proteins, and antibodies. These adaptive immune responses are tightly regulated and take several days or weeks to develop, and they have a memory function. The two parts of the immune system are linked, so that innate immune responses lead to activation of the adaptive immune system.

T-cell responses are crucial for anti-viral and anti-tumour immunity. A key player in the induction of T-cell responses is the dendritic cell (DC), which presents antigens to T cells, and activates them so that they attack virus-infected cells or tumour cells. These T cells act locally, i.e., cell-to-cell contact with virus-infected/tumour cells is required for the T cells to execute their functions. Thus, they have to be able to migrate to infected tissues/tumours. The focus of this thesis is on DCs and their functions in activating antigen-specific immune responses. The ultimate goals of this research are potential therapies that enhance anti-viral and anti-tumour immunity and possibly lead to tumour eradication.

T cells

Differentiation of naive T cells

Naive CD4⁺ and CD8⁺ T cells that have not yet encountered an antigen can develop into different subsets of T cells when they encounter an antigen-presenting cell, e.g., a DC. The differentiation pathway is determined by the activation signals and cytokines that the DC provides, as well as the cytokines in the local environment [1]. Cytokines

are small molecules that are used by cells for communication with other cells (Greek, cyto-, cell; and -kinos, movement). Cytokines are secreted into the surroundings, and affect both adjacent cells and the secreting cell itself through binding to the cytokine receptor. Cytokines are often produced by cells in response to a pathogen or a danger signal, leading to the activation of immune cells and enhancement of the immune response. After encountering a DC, T cells undergo cell division and become either effector T cells or memory T cells. Naive CD4⁺ T cells, the so called T-helper 0-cells (Th0), differentiate into one of four different subsets of effector T cells. Depending on the activation signals, different transcription factors are activated and decide the fate of the T cell (Figure 1). Three of the CD4⁺ T-cell subsets are T-helper cells (Th cells), so-called because of their roles in assisting other cells to exert their functions. Th1 cells participate in cell-mediated immunity, which is essential for the control of intracellular pathogens, such as viruses and intracellular bacteria, and in anti-tumour immunity. Th2 cells participate in humoral immunity against extracellular pathogens by providing help to B cells to produce antigen-specific antibodies. Th17 cells play an important role in antimicrobial immunity at epithelial/mucosal barriers against Gram-negative bacteria, fungi, and some protozoa. The regulatory CD4⁺ T cells function in suppressing immune responses, so as to maintain self-tolerance and homeostasis [2].

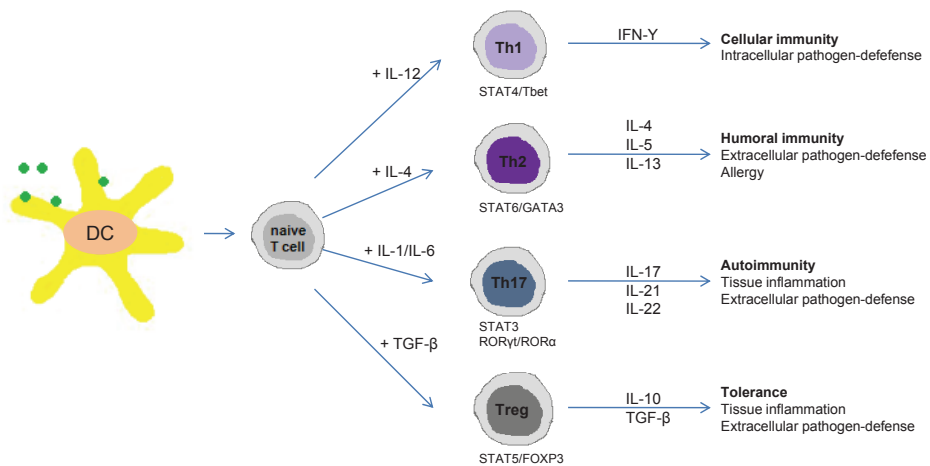


Figure 1. Differentiation of naive CD4⁺ T cells into different CD4⁺ T-cell subsets (adopted from [3]).

The naïve CD8⁺ T cells differentiate into cytotoxic CD8⁺ T cells or cytotoxic T lymphocytes (CTLs) upon contact with a CD4-activated DC. The CTLs are required for effective elimination of cells that are infected with intracellular pathogens (bacteria or viruses) or cells that are damaged/dysfunctional (e.g., tumour cells). Through contact with the target cells, the CTLs induce apoptosis (programmed cell death) of the target cells. CTLs release cytosolic granules that contain perforin, which generates holes in the membranes of the target cells. This allows granzymes and granzymes to enter the cell, thereby initiating a cascade of events that finally leads to apoptosis [4]. An alternative strategy to kill target cells is through the ligand-mediated pathway, whereby binding of the Fas ligand on the CTLs to the Fas molecules on the target cells leads to apoptosis of the target cell [5].

The Th1 response

IL-12 production by DCs is required to initiate the Th1 responses that are crucial for anti-viral and anti-tumour immunity. IL-12 induces T cells to proliferate and differentiate into Th1 cells and CTLs [6-9]. The Th1 cells produce IFN- γ , which is the main Th1 cytokine. Production of IFN- γ skews the immune response further towards the Th1 response, which is characterised by the activation of NK cell effectors, specific cytotoxic immunity, and macrophage activation [10]. T cells, NK cells, and macrophages are important for the elimination of tumours, and they are recruited to tumours by Th1 cells [11, 12]. IFN- γ increases the migration of immune cells to sites of inflammation by enhancing the expression of chemokines and adhesion molecules on leukocytes and endothelial cells. IFN- γ also stimulates the secretion of free radicals and perforin/granzymes by macrophages and CTLs, respectively, thereby enhancing their cytotoxic functions [13]. Furthermore, IFN- γ enhances the expression of MHC molecules on tumour cells and the expression of co-stimulatory molecules on DCs, leading to increased activation and recognition by T cells.

Migration and recognition of antigens by T cells

Chemokines and immune cell migration

All functional immune responses depend on the regulated migration of immune cells between compartments [14-16]. This process is called homing, and it depends on the expression of chemokines, chemokine receptors, and adhesion molecules. Chemokines are small peptides that belong to a superfamily of heparin-binding molecules secreted by a variety of cells [17]. The chemokines are promiscuous, in that they can bind to several different receptors. Different cells express different subsets of chemokine receptors (Table 1). Chemokines and chemokine receptors are expressed constitutively and guide leukocytes during immune surveillance, and they are

inducible, in that they recruit cells to sites of infection/inflammation [15, 18]. Chemokine secretion rapidly upregulates the expression of adhesion molecules on lymphocytes, which allows them to leave the blood and enter inflamed tissues through extravasation [14, 19]. Since T cells act locally, they have to be able to migrate to the site of infection/tumour to execute their effector functions. The chemokines measured in this thesis are expressed in inflamed tissues and tumours and can recruit DCs, CD4⁺ Th1 cells, cytotoxic CD8⁺ T cells, and NK cells. These include CCL3 (macrophage inflammatory protein 1 alpha, MIP-1 α), CCL5 (Regulated upon Activation, Normal T-cell Expressed, and Secreted, RANTES) and CXCL10 (interferon-gamma-induced protein of 10 kDa, IP-10) chemokines.

Table 1. Chemokine receptors and their expression patterns by different leukocyte subsets

<i>Cell attracted</i>	<i>Receptor</i>	<i>Chemokine binding</i>	<i>Reference</i>
Monocyte, macrophage	CCR2 and CX3CR1	CCL2, 3, 5, 7, 8, 13, 17, and 22	[17]
Mast cell	CCR1-5, CXCR2, and 4	CCL2, 5, and 11	[17]
Eosinophil	CCR3	CCL3, 5, 7, 11, 13, 24, and 26	[17]
Th2	CCR3, 4, 8	CCL11, 22, and 17	[17]
Antigen-specific Th1, CD8 ⁺ T cell and NK cell	CXCR3	CXCL10, 11, and CXCL9	[17]
Immature Langerhans cell	CCR6	CCL20	[15]
Immature blood CD11c ⁺ DC	CCR2	CCL2	[15]
Immature monocyte-derived DC	CCR1 and 5	CCL3 and 4	[15]
Immature CD34 ⁺ -derived DC	CCR6	CCL3, 5, and 20	[15]
Mature DC	CCR7	CCL 19 and 21	[15]

Antigen recognition and MHC molecules

A characteristic of the adaptive immune system is the use of antigen-specific receptors by T cells and B cells. T-cell receptors can only recognize an antigen that is presented as a processed peptide bound to a major histocompatibility molecule (MHC) on the

surface of another cell. The MHC molecules, or human leukocyte antigens (HLA), are encoded by a large, diverse family of genes. This diversity is important, as it enables the presentation of all possible antigens by MHC molecules. The MHC molecules are divided into MHC classes I and II. The CD8⁺ T cells only recognize peptides that are bound to MHC class I, which is present on all nucleated cells and binds peptides derived from cytosolic proteins, which can be either self-antigens or antigens from intracellular pathogens. The proteins are degraded to peptides by the proteasome [20], and delivered to the MHC molecules by the transporter associated with antigen processing (TAP) molecule [21]. The CD4⁺ T cells only recognize peptides that are bound to MHC class II, which is mainly present on antigen-presenting cells (DCs, B cells, and macrophages) and binds peptides derived from extracellular proteins that have been engulfed and degraded within the cells.

Dendritic cells (DCs)

General characteristics

DCs represent a sparse heterogeneous population of cells spread throughout the body, both in the peripheral and lymphoid tissues. The name comes from Greek *dendron*, meaning tree, and it refers to the dendrites that are characteristic of these cells. The common feature of DCs is their ability to capture and present antigens for naive T cells, which means that they are the main antigen-presenting cells (APCs) in the body. However, different DC subsets may have different antigen-presenting roles *in vivo* [22]. Immature DCs are not yet activated and express low levels of MHC class II. These cells circulate through the tissues into the lymphoid organs, where they present both self- and non-self-antigens [23]. The DCs are activated by a variety of signals emanating from microorganisms, inflammation, and tissue disruption [23-26]. Activation causes the DC to: a) mature into a highly efficient APC; and b) simultaneously migrate to lymphoid tissue, where it arrives fully equipped to induce a specific immune response.

Different types of DCs

A DC in the steady-state can be categorised as either a plasmacytoid DC (pDC) or a conventional DC (cDC) [27]. These different subsets have different migration patterns, express different receptors, and have slightly different functions. Another subset, the monocyte-derived DC (moDC), appears to exist only under inflammatory conditions, i.e., not in the steady-state.

The pDCs are also called natural interferon-producing cells, since they produce high levels of IFN- α upon viral infection. As they circulate through the blood and lymphoid tissues, pDCs display a round plasmacytoid shape, whereas they acquire the dendritic phenotype and functions upon infection or inflammation [28]. The cDCs are found in the lymphoid organs and peripheral tissues, and can be either lymphoid-organ-resident DCs or migratory DCs [29]. The migratory DCs circulate *via* the afferent lymph from the tissues to the local lymph nodes, where they present peripheral antigens [27]. The resident DCs and blood-derived DCs in the lymphoid organs migrate from the blood, and have therefore not been in the periphery [27, 29]. Under steady state conditions, these DCs capture and present antigens in the lymphoid tissues and remain immature unless they receive activation signals. The existence of moDCs (inflammatory DCs or emergency DCs) *in vivo* is a matter of debate, since they are not found under steady state conditions [27, 29]. However, moDCs seem to differentiate from monocytes during infection or inflammation [30]. The moDCs are the most commonly used DCs in human *in vitro* studies, since they are easy to propagate *in vitro*. The growth of CD14⁺ monocytes in cultures that contain GM-CSF and IL-4 generates high numbers of DCs [31]. In murine studies, the DCs are usually generated from bone marrow cell populations that are depleted of MHC class II-negative cells and cultured for 2-4 days in the presence of GM-CSF [32]. This method generates DCs that resemble the human monocyte-derived DCs [27].

DC functions

Activation of T cells

Mature DCs are the only cells that can activate naive T cells. The priming of naive T cells by DCs occurs in the T-cell areas of the lymph nodes. Upon activation, the migratory DCs upregulate their expression of the lymphoid chemokine receptor CCR7 and migrate to lymph nodes [24]. During maturation, the DCs enhance their surface expression of MHC-peptide complexes. This provides the naive T cell with the first activation signal, i.e., the antigen-specific signal, resulting in the binding of the MHC-peptide complex to the T-cell receptor. The second activation signal is the co-stimulatory signal, which consists of binding of the costimulatory molecules CD80 and CD86 on the DC to the CD28 on the T cell. The enhanced expression of CD40 on the mature DC primes it for CD40 ligand stimulation provided by the CD4⁺ Th cells [8]. The interaction between the DC and T cell stimulates the T cells to produce IL-2, which is necessary for T-cell survival and proliferation [33, 34]. CD40 stimulation enhances IL-12 production by DCs (Figure 2). IL-12, the third activation signal, is required for the differentiation of Th1 cells and cytotoxic CD8⁺ T cells. It has been suggested that failure of CD8⁺ T cells to control viral and tumour growth is due to the absence of IL-12 [35, 36].

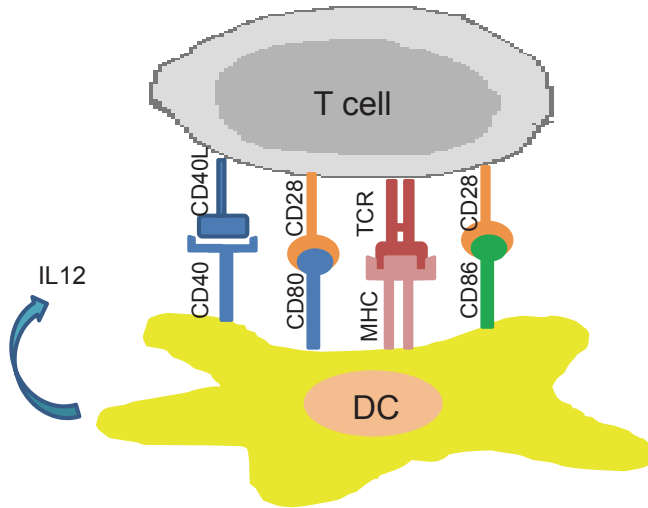


Figure 2. T-cell activation by DC.

Induction of tolerance/maintenance of peripheral tolerance

The interaction between a DC and a T cell can also lead to tolerance, depending on the type of DC involved and its activation state. DCs that are not fully activated and matured cannot provide the T cells with appropriate costimulation, which can lead to T-cell anergy. This means that the T cells cannot mount an immune response against their specific antigen(s) [37, 38]. Instead, there is induction of tolerance, which is a state of immune unresponsiveness to the specific antigen [39]. *In vivo*, DCs capture both self-antigens and foreign proteins without initiating an inflammatory immune response, and in the steady state, DCs are likely to be tolerogenic APCs, thereby maintaining peripheral tolerance to self antigens [29, 40]. The enzyme indoleamine 2,3-dioxygenase (IDO) is produced by many different cells, including murine and human DCs. IDO catalyses the metabolism of tryptophan, and one of the metabolites is kynurenine. IDO plays an immunoregulatory role in mice [41]; it prevents rejection of the fetus [42], and inhibits immune-mediated rejection of tumours [43]. The production of IDO is induced during the maturation of moDC *in vitro*, and its metabolic activity results in the inhibition of T-cell proliferation [44]. IDO and the tryptophan catabolic pathway appear to mediate immune tolerance in humans also [45]. Activated human T cells induce IDO production from DCs, which suggests that IDO downmodulates human immune responses [46]. IDO expression occurs in

tumours and in the DCs from tumour-draining lymph nodes, which implies that tumour-induced IDO expression mediates tolerance to tumour antigens [45]. It has also been suggested that IDO production by DCs negatively affects the success of therapeutic DC vaccination against cancer [46].

Toll-like receptors (TLRs)

In 1989, the immunologist Charles Janeway introduced the infectious-nonself model, which explains how the immune system distinguishes between foreign and self antigens. He proposed that APCs are activated and initiate immune responses by recognising conserved pathogen-associated patterns (PAMPs) on microorganisms [47]. These PAMPs are recognised by the pattern recognition receptors (PRRs) present on cell membranes. The Toll-like receptors (TLRs) belong to the PRR family and have the ability to sense and bind PAMPs from different microorganisms [48]. Currently, there are 12 known human TLRs, which are located extra- or intracellularly, depending on ligand specificity (Table 2) [49, 50]. TLRs bind both microbial and endogenous ligands. The latter arise from tissue damage and include heat-shock proteins, components of the extracellular matrix, and endoplasmic reticulum proteins [48]. The ‘danger model’, which was introduced by the immunologist Polly Matzinger in 1994, explains why TLRs bind and are activated by endogenous ligands [51]. In this model, it is suggested that the immune system is activated by danger/alarm signals from injured cells.

Table 2. The Toll-like receptors

<i>Group</i>	<i>Ligand source</i>	<i>Location</i>
TLRs 1, 2, 4 and 6	Lipids - bacteria, fungi	Cell surface
TLRs 5 and 11	Protein ligands - bacteria	Cell surface
TLRs 3, 7, 8, and 9	Nucleic acids - bacteria, viruses	Intracellular compartments - endosomes, lysosomes

Many of the TLRs are expressed on the main sentinel cells of the innate immune system, i.e., epithelial cells, phagocytic cells, and the various DC subsets (Table 3). Activation of the TLRs on these cells initiates antimicrobial and inflammatory

responses through TLR signalling [24]. TLR signalling activates different intracellular signalling pathways depending on the cell type and specific TLR involved, the most common pathway being that leading to activation of NF- κ B. The intracellular pathways give rise to a variety of responses in the activated cells (Figure 3) [24].

Table 3. Immune cells and their TLR expression patterns

<i>Human cell type</i>	<i>TLR mRNA expression</i>
Neutrophil	TLRs 1-2, 4-10
NK cell	TLRs 1-10
Eosinophil	TLRs 1, 4, 7, 9, 10
Mast cell	TLRs 1, 2, 4, 6
Monocyte	TLRs 1, 2, 4, 5, 6, 8
Monocyte-derived DC	TLRs 1, 2, 3, 4, 6, 8
Myeloid DC	TLRs 1, 2, 3, 5, 6, 8
pDC	TLRs 6, 7, 9

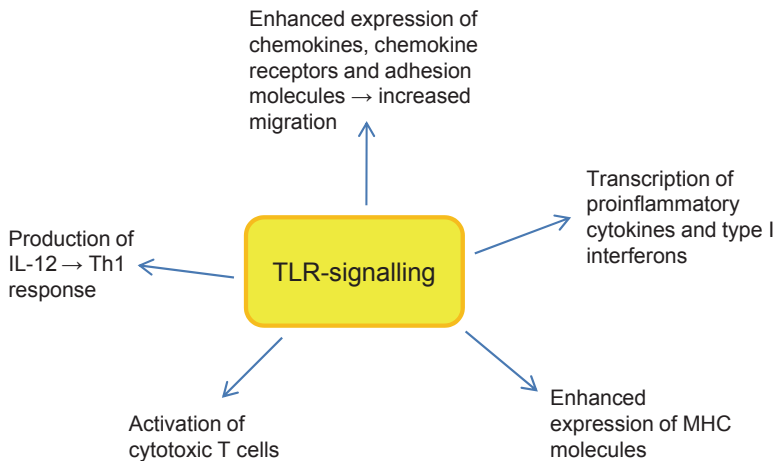


Figure 3. Pleiotropic effects of TLR signalling.

The understanding of cancer – a historical overview

The ancient Egyptian believed that it was the Gods who caused cancer, and the first known descriptions of cancer are from papyrus texts from the 16th century BC (the Edwin Smith Papyrus). It describes how breast tumours were treated with a hot instrument that destroyed the tissue. Skin tumours were treated in ancient Egypt by removing the affected tissue. Both the words ‘cancer’ and ‘oncos’ derive from the Greek language. Karkinos (later translated to Latin, carcinos or carcinoma, meaning cancer) means crab, a shape that reminded the famous Greek physician Hippocrates from Kos (460 BC - 370 BC) of how non-ulcer and ulcer-forming tumours could appear. The word ‘oncos’ means swelling, and this term was used by the Roman physician Galenos (129 AD - 199 AD) to describe tumours. Galenos proposed the theory that cancer was a substance that spread from the blood into the tissue, and that it was caused by a misbalance in the body fluids. This theory was accepted as the cause of cancer until the 18th century.

In the 17th and 18th centuries, knowledge of cancer was significantly improved by an Italian renaissance physician, Giovanni Morgagni of Padua (1682-1771), who by performing autopsies linked the clinical course of illness to pathological findings. His student, Antonio Scarpa (1752-1832), continued this work and wrote several books about cancer. In the 18th century, cancer started to be considered as a local tissue-specific disease independent of body fluids. The pioneer of modern pathology, Rudolf Virchow of Germany (1821-1902) used the modern microscope to examine surgically removed body tissues, so as to make a precise diagnosis. He defined tumours as aggregates of new cells derived by cell division.

The discovery in the 1950s of the chemical structure of DNA by James Watson, Francis Crick, Maurice Wilkins, and Rosalind Franklin led to the understanding of how genes work, and how mutations affect cells. It soon became clear that the damage caused to DNA by chemicals or radiation and the introduction into cells of new DNA sequences by viruses could promote the development of cancer. In the 1970s, oncogenes and tumour suppressor genes were discovered.

Viruses and cancer

Around 20% of all cancers worldwide are caused by infectious agents, commonly viruses. Over the past 15 years, several viruses have been identified as potential cancer-causing agents, and probably others remain to be identified. Peyton Rous, who

received the Nobel prize in 1966, described already in 1911 how a sarcoma could be transmitted between chickens by inoculation of a cell-free filtrate [52]. In the 1930s, Rous investigated the connection between papillomavirus and cancer in rabbits [53]. Later, several different viruses that could cause cancer in animals were discovered. In 1964, the first virus that could cause cancer in humans, the Epstein-Barr virus, was identified; today, the research area of cancer-inducing viruses is a hot topic [54]. Some of the viruses that have been implicated in cancer in humans are listed in Table 4.

Table 4. Cancer-causing viruses

<i>Virus</i>	<i>Type of cancer</i>	<i>Reference</i>
Epstein-Barr virus	Burkitt's lymphoma	[55, 56]
Hepatitis B virus	Liver cancer	[57, 58]
Hepatitis C virus	Liver cancer	[59]
Human T-cell lymphotropic virus type 1	T-cell leukaemia	[60, 61]
Human herpesvirus type 8	Kaposi's sarcoma	[62]
Papillomaviruses	Cervical cancer, head and neck cancers, anogenital cancers, lung cancer ?	[63]
Merkel cell polyomavirus	Merkel cell carcinoma	[64]

Human papillomavirus and cervical disease

Human papillomavirus (HPV)

Papillomaviruses are double-stranded DNA viruses, which in 2004 were assigned to a taxonomic family of their own, the Papillomaviridae [65]. The papillomaviruses are species-specific, and probably occur in most mammals and birds. The most widely studied of these viruses are the human papillomaviruses (HPVs). Currently, there are more than 100 known HPVs [65, 66], some of which are listed in Table 5. About 40 of the HPVs are viruses that infect the anogenital mucosa. These can further be divided into high-risk and low-risk types, depending on their propensity to cause malignancy [67]. Low-risk HPV6 and HPV11 are associated with benign genital warts (*condyloma accuminata*) [68]. The high-risk HPV types cause lesions that can develop into cervical, penile, anal, vaginal and head-and-neck cancers. Since the 1840s, various attempts have been made to connect the prevalence of cervical cancer to sexual behaviour [69-74], with studies comparing the cervical cancer incidences for nuns, prostitutes, married women, and unmarried women. However, none of these

studies managed to establish unambiguously a relationship between sexual contact and cervical cancer, until the 1960s, when it was suggested that viral infection might underlie this life-threatening malignancy [75, 76]. At that time, the causative agent was thought to be Herpes simplex virus type 2. In 1974, Harald zur Hausen was the first to hypothesise that cervical cancer could be caused by HPV [77]. For this discovery, he received the Nobel Prize in Medicine in 2008.

Table 5. Common HPVs with different tropisms

<i>HPV group</i>	<i>Location(s)</i>	<i>HPV type</i>
Cutaneotropic	Skin (warts) and epithelium (tumours)	HPVs 1, 4, 5, 8, 41, 48, 60, 63, and 65
Mucosotropic	Mucosa of the anogenital tract	HPVs 6, 11, 13, 16, 18, 26, 31, 32, 33, 34, 35, 39, 42, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 64, 66, 67, 68, 69, 70, and 73
Cutaneotropic/ mucosotropic	Cutaneous or mucosal tissues and lesions	HPVs 2, 3, 7, 10, 27, 28, 29, 40, 43, 57, 61, 62, and 72

Functions of the HPV proteins

The HPV genome consists of an 8-kb closed-circular, double-stranded DNA. It encodes eight proteins, including the early proteins E1, E2, E4, E5, E6, and E7, and the late structural proteins L1 and L2 [68]. The expression of the viral gene products is closely regulated during the life-cycle of the virus. The time points at which the different genes are transcribed are dependent upon the HPV type that causes the infection, as well as the severity of the infection [78, 79].

The early proteins are regulatory proteins that are linked to viral genome persistence, DNA replication, and activation of the lytic cycle [68]. E1 and E2 are required for HPV replication [79]. E1 catalyses the unwinding of DNA and initiates viral replication. E2 has an important function in regulating the expression of E6 and E7. Low levels of E2 are involved in replication, while high levels of this protein repress the replication and functions of E6 and E7. E4 is a non-structural protein that is expressed in the upper layers of the infected epithelium. Its function is not yet fully understood, although it seems to allow the virus to complete its life cycle. Little is known about the function of E5 during normal HPV infection, although it appears to function during the vegetative phase of the cell cycle. E6 and E7 are necessary to maintain the cell in the cell cycle. L1 and L2 together form the viral capsids [68].

HPV infection of the epithelium

In normal uninfected squamous human epithelium, the basal cells are undifferentiated and able to divide. After division, one of the daughter cells begins to migrate towards the upper layers of the epithelium. During migration, the cell exits the cell cycle and undergoes terminal differentiation. It produces keratin, which eventually breaks the nuclear envelope, with the consequence that the cell stops working and becomes a keratin-filled sac [79, 80]. The HPV life cycle is linked to the differentiation stage of the host cell. The proliferative phase of the HPV16 virus life-cycle starts with infection of the basal layers of the epithelium by HPV virions, which probably enter through micro-abrasions in the squamous epithelium [79, 80]. In the basal keratinocytes, the early HPV genes E1, E2, E5, E6, and E7 are expressed and the viral DNA replicates as a low-copy viral episome, thereby generating 50-100 virus episomes per infected cell. When the basal cells divide, the viral episomes are segregated equally among the daughter cells. During the migration of the daughter cells towards the surface, the viral proteins block the cells from leaving the cell cycle, and normal terminal differentiation is delayed. Thus, the cell remains in the cell cycle, so to as produce more virions. In the upper layers of the epithelium, the viral genome is replicated, and E4 and the late genes L1 and L2 are transcribed. The genomes are packed into new capsids, which are then released from the cell.

HPV can exist in a latent state in the basal epithelial cells, with a low production level of virions. Reactivation of the virus from the latent state can occur, for instance, following a decline in immunosurveillance [78].

Different types of cervical cancer

The two main types of cervical cancer are: 1) squamous cell carcinoma, which accounts for about 75% of cervical cancers; and 2) adenoma carcinoma, which accounts for 15-20% of cervical cancers [81]. Squamous cell carcinomas often start to develop in the transformation zone that connects the endocervix and ectocervix. The cancer cells are flat, thin cells that are derived from the squamous cells that cover the epithelium of the ectocervix. Adenocarcinomas develop from the mucous-producing glands of the endocervix. Adenosquamous carcinomas are a mixture of these two types of carcinomas, consisting of both squamous cells and gland-like cells. Other types of cancer can also originate in the cervix, e.g., melanoma, sarcoma, and lymphoma.

Analysis of viral infection

Currently, no standardised serological assay is available to assess neutralising antibody responses to HPV. It is difficult to use serology to test for the presence of antibodies in the diagnosis of ongoing HPV infection or previous HPV infection, as around 50% of infected individuals do not seroconvert. Instead, diagnosis is often made on the basis of PCR detection of viral DNA in cervical cells.

Screening for cell transformation

The pre-malignant phase of HPV infection is often discovered in Papanicolaou smears (Pap smears) taken during a routine control at a primary healthcare facility. Cytological examination of these exfoliated cervical cells reveals abnormal changes in the cell morphology. These changes are confirmed by histological examination of cervical tissue biopsies. Early growth on the cervix of abnormal cells, which have the potential to progress to cancer, is often referred to as ‘cervical dysplasia’ or ‘cervical intraepithelial neoplasia’ (CIN). Neoplasia is a Greek word for new growth, and it refers to the observed abnormal cell growth. These cells are not considered to be malignant, since they remain on the surface of the cervix and are not yet invasive. Depending on the extent to which epithelial differentiation is disturbed, these pre-malignant lesions are classified as CIN I (low-grade neoplasia), CIN II (moderate neoplasia) or CIN III (severe neoplasia) [79, 82]. CIN IV is regarded as cancer *in situ*. In addition, the HPV-induced lesions can be classified as low-grade or high-grade squamous intraepithelial lesions (LSIL is equivalent to CIN I; HSIL is equivalent to CIN II-III) [82].

Epidemiology

Infection with HPV is the most common sexually transmitted disease in the world [83]. The natural history of HPV infections is dynamic, with frequent acquisition of both low-risk and high-risk types [84]. Approximately 75% of all sexually active individuals get infected with HPV at least once during their lifetime [85]. Most HPV infections are subclinical and heal within 12 months. In the absence of regression of the infection, the cervical lesions may progress to cancer. However, this is a rare event considering the high prevalence of HPV infections in the general population. HPV high-risk types were estimated to be responsible for 5.2% of all cancers worldwide in the year 2002 [86]. These cancers include cancer of the cervix, penis, vulva, vagina, anus, mouth, and oropharynx. The second most common malignancy in women worldwide is cervical cancer, which accounts for approximately 9.8% of all female cancers [87]. Cervical cancer is the third leading cause of mortality due to cancer worldwide, and has the highest rank in the less-developed world [88]. Around 291

million women in the world are estimated to be infected with HPV [83]. Approximately 450000 of these women will develop cervical cancer each year over the next few decades [83, 89], and around 230000 women die each year of cervical cancer [90, 91].

The prevalence of HPV in women with normal cytology has been estimated to be 10.4% [83]. HPV infection is more common in less-developed parts of the world, with Africa having the highest prevalence (22.1%) and Europe (8.1%) and Asia (8.0%) having the lowest. The prevalence of HPV is highest worldwide in women under 25 years of age [83]. Thereafter, the prevalence declines continuously with age. However, a second rise in prevalence is seen in the age range of 45-54 years and older in Europe and Africa, and in the age range of 35-44 years and older in North, Central, and South America [83].

HPV16 is the most common HPV type worldwide, infecting 23.3% of the 291 million HPV-infected women in the world, followed by HPV18, which infects 8.5% [83]. The prevalences of HPV16 infection are: 2.5% for women with normal cytology [83]; 45.4% for women with high-grade squamous intraepithelial lesions; and 55% for women with invasive cancer [92].

Risk factors for cervical cancer

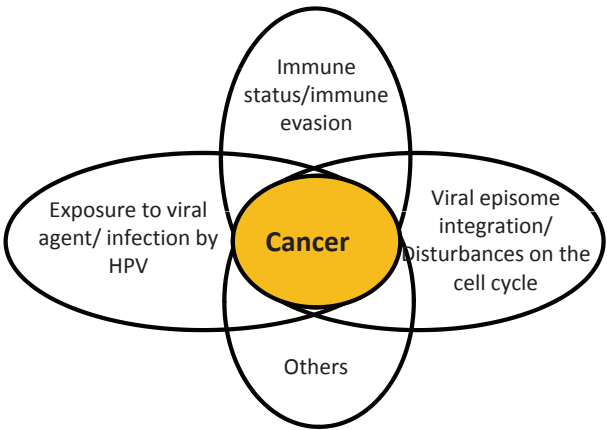


Figure 4. Risk factors for cervical cancer.

Exposure to viral agent/infection by HPV

Several risk factors are involved in the development of cervical cancer (Figure 4). The greatest risk factor for cervical HPV infection is being sexually active. Early sexual debut and multiple sexual partners are factors that contribute to higher exposure to the virus. Another significant risk factor for acquiring cervical HPV is having a high-risk partner. Indeed, male sexual behaviour is an important factor in the spread of genital HPV. Sexual contacts with prostitutes by husbands may be more important than the number of sexual partners in a woman's lifetime [93, 94].

Infection with high-risk HPV types is a causal and necessary factor for cervical cancer [87]. Only a minority of high-risk HPV infections will cause cervical cancer precursor lesions, and of these lesions, only a small fraction causes cervical cancer [67]. Progression to malignancy often takes several years, which means that persistency of the infection is a prerequisite [87]. It has been suggested that multiple concomitant HPV infections contribute to the pathogenesis by acting synergistically in cervical carcinogenesis [95]. Several studies show that multiple infections are more common in women with a higher degree of cervical disease, as compared with control subjects who have normal cytology or lower grade of disease [96-98].

There appears to be a connection between high viral load in high-risk HPV infections and the risk of progression in cervical disease [99]. Several studies have found this association for HPV16 infection [100, 101]. Other studies have failed to find any such connection, but instead have shown varying loads of HPV16, 18 or 52 DNA in the cervical cells of different disease grades [102].

Immune status and immune evasion

Several factors contribute to the development of HPV-linked cancer. Although avoidance of the immune system and disruption of the cell cycle by HPV are important aspects, it is likely that many other risk factors are involved. The cell-mediated immune system is important in controlling HPV infections. The lack of proper CD4⁺ T-cell responses increases the risk of developing HPV-induced malignancy [103]. It is clear that genetic susceptibility of the host influences the likelihood of developing cervical cancer. Some HLA class I and II haplotypes appear to influence HPV infection and its progression, although to date, no strong associations have been demonstrated when the HLA allele frequencies of women with HPV-induced cervical disease have been compared with those of healthy controls [104-107].

The use of oral contraceptives has been linked to an increased risk of developing cervical cancer [108, 109], as well as to a four-fold increase in the risk of cervical carcinoma in HPV-positive women [110]. Although the reason for this is not fully clear, several contributory factors have been proposed, such as: a) effects on the epithelium due to hormones [111]; and b) effects on the immune system [112, 113]. I believe that the use of oral contraceptives reduces condom use, which may lead to more HPV infections. High parity is another cofactor for progression to cancer, which may be coupled to the effects of hormones [114, 115]. The concentrations of oestrogens and progesterone increase during pregnancy, which may alter the transformation zone of the cervix, making it more susceptible to HPV [115].

The life-cycle of HPV and the location of the infection are important factors in terms of the ability of HPV to evade the immune system. HPV infects keratinocytes, which have a short life-span. Therefore, new virions are released in a non-lytic way from sloughed-off cells at the epithelial surface. This release of virions does not result in inflammation, which means that the innate immune responses are avoided [116]. Furthermore, the life-cycle of the virus is limited to the keratinocyte differentiation program, meaning that the highly immunogenic capsids are produced in the outer layer of the epithelium, which has no immune cells. Even though the early viral proteins are expressed in cells that are exposed to APCs, they appear to be ignored by the cells of the immune system [116]. The viral oncoproteins downregulate several important immune responses, affecting antigen presentation, innate anti-viral immune responses, and anti-viral specific responses, some of which are listed in Table 6.

Table 6. Viral modulation of important immune mechanisms

<i>Viral proteins/genes involved</i>	<i>Effect on host cell</i>	<i>Downstream effect</i>	<i>Reference</i>
E6, E7	TLR9↓	Block recognition of viral CpG-rich DNA	[117]
E6, E7	IRF-3 ^a ↓, IRF-1 ^b ↓	Block transcription of the anti-viral cytokine IFN-β	[116]
E6	IL-10↑	Suppresses of cellular immune responses	[118-120]
E7	TAP ^c ↓	Downregulates expression of MHC class I → no recognition by CTL	[121-123]

a: IRF-3: interferon regulatory factor 3; b: interferon regulatory factor 1; c: transporter associated with antigen processing

Viral episome integration and disruption of the cell cycle

The integration of the virus episome into the host chromosome occurs in most cases of advanced HPV16-induced cervical disease. The frequency of integrated DNA increases with the severity of disease; it is rarely seen in CIN, whereas it is often observed in invasive cervical cancer [124, 125]. The integration of HPV16 DNA into the chromosome correlates with increased expression of E7 and the acquisition of a growth advantage, which may explain why epithelial precursor cancer cells grow better [126]. In contrast, HPV18 is most often found in the episomal form, even in advanced disease [124]. Integration of the HPV episome into the host chromosome often disrupts the E2 gene. This means that the cell loses control of the expression of the two main oncogenes, E6 and E7, which are normally regulated by E2 [124, 127]. This may lead to the overexpression of E6 and E7, the production of fewer virions, and an even less-differentiated epithelium [128] (Figure 5). E6 and E7 interfere with the two important tumour suppressor proteins, protein 53 (p53) and Retinoblastoma protein (pRb), thereby causing genetic instability and cell cycle dysregulation [68]. pRb binds to the cellular transcription factor E2F, thereby preventing the cell from entering the S phase [78]. HPV E7 binds to pRb and disrupts the pRb-E2F complex. When E2F is released, the cell enters the S phase. Activation of p53 is normally induced by DNA damage, and leads to either cell cycle arrest in the G1 stage or apoptosis. Binding of HPV E6 to p53 results in rapid proteasomal degradation of p53. Thus, E6 contributes to the development of cancer, since errors in the host cell DNA go unchecked [78]. E6 complements the role of E7, and seems to prevent apoptosis in response to the unscheduled S-phase entry mediated by E7.

MYC is a proto-oncogene that regulates cell functions, such as apoptosis and differentiation [129]. MYC expression is often found in many different tumour types [130]. HPV16 or HPV18 DNA is usually inserted in the host chromosome at the MYC locus, which can cause insertional mutagenesis and lead to overexpression of the proto-oncogene MYC [130].

Other risk factors

There probably are several other co-factors for progression to cancer. An increased risk for cervical cancer *in situ* has been related to smoking [131-133]. Evidence for the direct effects of smoking includes the discovery of constituents of tobacco smoke in cervical mucous [134] and higher levels of smoking-related DNA damage in CIN and cervical cancer epithelium, as compared with normal epithelium [135]. Low socio-economic status has also been reported as a risk factor for the development of cervical cancer. Cervical cancer has also been associated with low educational level, leading to

a lower frequency of Pap smears and more frequent visits to prostitutes by the husband [94], as well as poor genital hygiene [114, 136, 137].

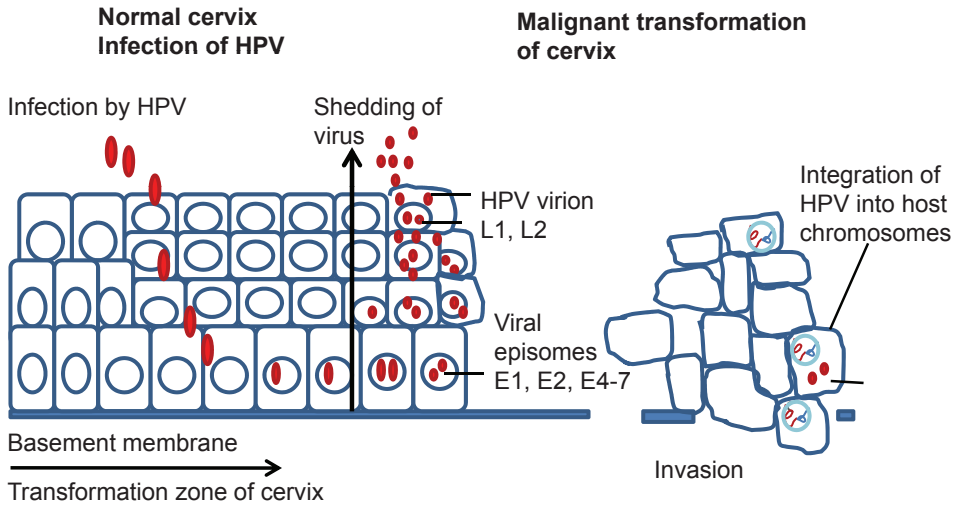


Figure 5. Malignant transformation of cervix

Prophylactic vaccines

Vaccination as a means of protecting against an infectious disease originated in 1789, when Edward Jenner published an article describing a vaccine against smallpox. Since then, many different infections have become preventable by prophylactic vaccines, including certain HPV infections. Two different prophylactic HPV vaccines have been approved by national regulatory agencies in several countries [138]. Both vaccines are considered highly immunogenic and are composed of HPV L1 proteins that spontaneously assemble into virus-like particles. Upon injection, they induce neutralising antibodies to the viral capsids. Both Gardasil (Merck & Co) and Cervarix (GlaxoSmithKline Biologicals) protect against the two most common cancer-causing HPV types, HPV16 and HPV18. Gardasil also targets HPV6 and HPV11 and protects against genital warts [138]. Cervarix provides cross-protection against HPV45 and HPV31 [139].

Therapeutic cancer vaccines

A successful therapeutic cancer vaccine must eradicate the tumour and provide long-term protection against metastases. To date, few clinical trials of therapeutic cancer vaccines have been successful in terms of promoting tumour regression and long-term survival, which are the ultimate goals in treating cancer patients with therapeutic vaccines [140]. In therapeutic cancer vaccination, activation of both antigen-specific CD4⁺ and CD8⁺ T cells is desirable, since CD4⁺ T cells are needed for robust long-term tumour immunity [13, 141, 142] and CD8⁺ T cells are crucial for eradication of tumours.

For the control of HPV infections, cellular immune responses are essential, whereby CD4⁺ T cells appear to be of vital importance [103, 143-145]. Sustained activation of CD4⁺ T cells correlates with HPV disease regression. In a study of women with cervical neoplasia, lack of CD4⁺ T-cell reactivity was observed in patients who were more likely to have progressive disease [146]. Furthermore, partial or complete failure of CD4⁺ T-cell function, i.e., antigen-specific proliferation and/or the secretion of pro-inflammatory cytokines, has been demonstrated in patients with HPV16-induced cervical disease [119]. The vaccines that are currently available to prevent HPV infection do not have any effect on individuals who are already infected [138]. Although early-stage cervical tumours can be cured by surgery or radiation therapy, up to 50% of the patients with positive lymph nodes are at risk of developing recurrent disease with poor treatment outcomes [147]. Therefore, there is an urgent need for therapeutic vaccines against HPV and cervical cancer for women who are at a risk of developing malignancies or who already have cervical cancer. Several different strategies have been used to develop a therapeutic vaccine for patients with oncogenic HPV-induced lesions. These include live vector vaccines (viral and bacterial vectors), peptide/protein vaccines, nucleic acid vaccines (DNA vaccines and RNA replicon vaccines), cell-based vaccines (DC-based and tumour cell-based vaccines, and combinations thereof) [148].

Dendritic cell vaccine

DCs play an important role *in vivo* in activating T cells, and they can initiate effective immune responses and memory T cells. Therefore, the DC is a good candidate for a therapeutic cancer vaccine. The aim in developing a DC cancer vaccine is to generate high numbers of DCs, expose them to tumour antigens, and identify DCs that present tumour-derived epitopes on HLA molecules. These DCs can activate *in vivo* antigen-specific T cells, so as to turn them into effector cells that enter the tumour tissues, recognize the tumour antigens, and kill the tumour cells. Compared to the traditional

vaccination methods, a DC vaccine might be better at eliciting the appropriate type of immune response, since the DC can be manipulated *in vitro* to elicit the optimal immune response *in vivo* [149, 150]. Furthermore, clinical trials have shown that DC vaccines are safe with few treatment-related side-effects [147, 151, 152]. Several different parameters could be changed in DC vaccination protocols, including the method of generation of DCs, administration routes, and choice of antigens (Table 7) [153].

Table 7. DC generation methods, administration routes, and antigens in DC vaccines

		<i>Reference</i>
Type of DC vaccine	Autologous	[154]
	Allogeneic	[154]
Source of DC	Monocytes (GM-CSF, IL-4 <i>in vitro</i>)	[31]
	CD34+ HPCs ^a (GM-CSF, TNF- α <i>in vitro</i>)	[155, 156]
	Peripheral blood DCs (Flt3L <i>in vivo</i>)	[157]
	pDCs, cDCs	[158]
Antigen stimuli	Peptides	
	Proteins	
	Antigen-antibody complexes	
	mRNA, DNA	
	Viral vectors	
	Tumour cells and tumour cell lysates	[158]
Route of administration	Intravenous	[153, 158, 159]
	Subcutaneous	
	Intradermal	
	Intranodal	
	Intralymphatic	
	Intratumoural	

a: haematopoietic progenitor cells

Historical overview of therapeutic DC vaccines against cancer

In 1995, the first therapeutic DC vaccine was tested in mice with tumours [160]. The tumour cell lines used had well-defined CTL-recognized MHC class I-restricted epitopes, and immunodominant peptides were used as antigen. The DCs generated from bone marrow using GM-CSF and IL-4 were the most efficient in inducing CTL responses, and eradication of tumours was achieved in 80-100% of the cases. Two years later, in 1997, the first clinical pilot study of a therapeutic DC vaccine was

performed on four B-cell lymphoma patients using a tumour-specific idiotype protein as the antigen and moDCs [161]. Three out of four patients showed clinical responses. Since then, therapeutic DC vaccines have been tested in many different clinical trials (reviewed in [162]). The cancers that have been targeted include prostate cancer [163], hepatocellular carcinoma [164], renal cell carcinoma [165] and malignant gliomas [166] with a focus on melanoma [167-169].

DC vaccine for HPV-induced tumours

Cervical cancer is the only HPV-induced malignancy that has been targeted with therapeutic DC vaccination in clinical trials [147, 151, 152]. In these clinical trials, monocyte-derived DCs were loaded with HPV16 or HPV18 E7 as the antigen [147, 151, 152]. The HPV E7-loaded DC vaccine was reported to be safe and immunogenic for patients with early stage cervical cancer [147]. However, the clinical results have been limited. When tested in patients with late stage cervical cancer, no objective clinical responses were observed for this vaccine [151, 152]. Therefore, there is ample room for improvement of DC vaccination protocols.

Limits and challenges

Several problems need to be overcome prior to the general use of DCs as therapeutic vaccines in clinical trials [170]: a) late-stage cancer patients are heavily immune-suppressed due to radiation, chemotherapy, and heavy tumour burdens; b) few of the injected DCs actually migrate to the lymph nodes; c) the quality of the DCs has to be carefully controlled so that the right type of T-cell response is activated; d) systematic evaluation of protocols for generating DCs for clinical use are needed; and e) the appropriate antigen has to be used.

Another challenge that remains to be overcome in developing a therapeutic vaccine against HPV-induced malignancy is the immunosuppressive and anti-inflammatory environment created by cervical tumours [116, 171-173]. Since several pathways of the immune defence against tumours are abrogated, the immune system fails to recognize and eradicate the tumour cells. Without danger signals, the DCs in tumours may become improperly activated, which may lead to deficient T-cell activation or T-cell anergy. Chemokine production by tumours can be inhibited, thereby preventing influx of effector cells. Furthermore, tumour cells hide from effector cells by down-regulating their expression of MHC molecules [174]. Thus, the challenges in creating an effective therapeutic vaccine against HPV-induced disease are to enhance the T-cell activating capacity of the DC, to make the tumour cells visible to the immune system, and to induce the infiltration of effector cells into the tumour.

Cholera toxin as an adjuvant

Cholera toxin (CT), which is a toxin produced by the bacterium *Vibrio cholera*, causes severe diarrhoea in humans [175]. CT is less toxic in experimental animals, where it is used as a powerful mucosal adjuvant [176, 177]. CT consists of two subunits. The A subunit (CTA) has the catalytic activity and induces ADP-ribosyltransferase activity, which is important for the adjuvant effect [178, 179]. The B subunit (CTB) is the cell-binding part, which binds to its receptor GM1 present on all nucleated cells. CT enhances the expression of MHC class II molecules and co-stimulatory molecules, such as CD80, CD83, and CD86, on exposed DCs, which are important for efficient antigen presentation [180], [181-183]. CT has been reported to induce Th1 and Th2 cells [177, 181, 184], as well as regulatory T cells [185]. DCs treated with CT show enhanced expression of chemokine receptors CXCR4 and CCR7, which are necessary for homing to the lymph nodes [184, 186]. The *in vivo* mechanism for CT adjuvanticity in mice involves the migration of DCs to the T-cell zone in the spleen [183].

We have investigated the role of CT or CTB as a carrier and adjuvant in several different models, both *in vitro* in animals and *in vivo* in the human system, using DCs exposed to CT- (or CTB-)conjugated antigens [182, 187-189]. The favourable effects observed for the antigen-presentation and maturation of DCs are dependent upon the CTB subunit enhancing the uptake and presentation of antigens by binding to its receptor, as well as the adjuvant effects induced by the catalytic CTA subunit.

AIMS

The overall aim of my thesis was to develop and evaluate a possible treatment against HPV-induced cervical disease. For this purpose, the specific aims were:

- To investigate if intravaginal treatment of mice with TLR-ligands can upregulate the expression of MHC molecules in the genital epithelium and enhance recruitment of immune cells to the vaginal mucosa through induction of chemokine production.
- To evaluate the immunotherapeutic potential of using dendritic cells pulsed with cholera toxin-conjugated HPV16 E7 in combination with locally administered TLR ligands for the therapeutic treatment of HPV16 E7-expressing tumours.
- To examine if cholera toxin as a carrier/adjuvant can enhance the T-cell immune responses to E7-pulsed dendritic cells *in vitro* in women with HPV-induced cervical dysplasia.
- To study if cholera toxin induces the expression of the immunoregulatory enzyme indoleamine 2,3-dioxygenase in exposed dendritic cells.

MATERIAL AND METHODS

Recombinant HPV16 E7

Protein preparation

T7-tagged recombinant protein HPV16 E7 was produced from *Escherichia coli* strain BL21 carrying the pET-21a(+) vector (a kind gift from Dr. Wen L. Dong, International Agency for Research on Cancer, Lyon, France). Expression of the protein was initiated by adding isopropyl- β -D-thiogalactoside to an overnight culture of the bacteria in LB medium with ampicillin. The T7-E7 fusion protein was purified with the T7 Tag Affinity Purification kit, and detected as an 18.5-kDa band on SDS-PAGE. The protein solution was dialysed against PBS and concentrated by sprinkling solid polyethylene glycol over the dialysis tubing.

Conjugation to CT

The E7 protein was chemically coupled to CT using *N*-succinimidyl (3-[2-pyridyl]-dithio)propionate as a bifunctional coupling reagent [190]. The conjugated material was quantified and purified by FPLC gel filtration (Superdex 200 16/60 column; Pharmacia Biotech, Sweden) using the Biologic Workstation FPLC system (Bio-Rad Laboratories, Hercules, CA, USA). The purified conjugate contained two or four CT molecules per HPV E7 molecule. The conjugate was analysed in a GM1-ELISA and was shown to have retained GM1-binding activity.

Murine studies

Mice

Female 6–10-week-old C57BL/6 mice were purchased from B&K Universal, Sweden. We also obtained sex- and age-matched CD8^{-/-} mice [191] from Jackson Laboratories, Germany. Mice were housed in the animal facility at the Department of Rheumatology and Inflammation Research, University of Gothenburg, Sweden. The studies were approved by the Ethical Committee for Animal Experiments, Gothenburg, Sweden.

Intravaginal TLR treatment

We used the TLR agonists CpG-ODN (5'-TCCATGACGTCCTGATGCT-3' with a phosphorothioate backbone), Imiquimod acetate, and poly(I:C) (synthetic double-stranded RNA, polyinosinic-polycytidylic acid salt). All the compounds were dissolved at 1 mg/ml in PBS and stored at -20°C. The mice were pre-treated with

subcutaneous injections of Depo-Provera to synchronise their sex hormone cycles, 5 days before intravaginal administration of PBS or the TLR agonist.

Bone marrow-derived DCs and pulsing with antigen

To generate DCs, bone marrow cells from the tibia and femur were depleted of T cells and B cells and cultured in tissue culture medium that was supplemented with recombinant murine GM-CSF. DCs were harvested after 6 days of culture and more than 90% were CD11c-positive. The DCs were washed with PBS, and pulsed for 2 h at 37°C with HPV16-E7 or CT-E7 at a concentration of 0.05 μ M of E7 in culture medium, or with the equivalent (mol:mol) ratio of CT. After pulsing, the DCs were extensively washed to remove any unbound antigen prior to use.

The TC-1 tumour cell line and therapeutic tumour treatment

As a model system for HPV-induced cancer, we used the tumour cell line TC-1, which is derived from C57BL/6 lung tumour epithelial cells and co-transformed with HPV16 E6/E7 and activated H-ras. [192]. TC-1 cells can be cultured in normal culture medium, and we always used cells that had been split 1:2 on the day before inoculation. TC-1 cells (5×10^4) were injected intradermally (ventrally above the sternum) into the mice. Two days later, 2×10^6 antigen-pulsed DCs were injected intravenously into the tail vein. This vaccination was repeated twice at weekly intervals. TLR ligands were administered intratumourally on the same occasions. Tumour growth was assessed 2–3 times per week. Animals were sacrificed once the tumour reached a diameter of 1.25 cm in the untreated control mice.

Cytotoxic T-cell assay

Mice were vaccinated with antigen-pulsed DCs twice, 1 week apart. Two weeks later, spleen mononuclear cells were isolated and restimulated with a H-2Db-restricted CTL epitope from HPV16 E7⁴⁹⁻⁵⁷ (RAHYNIVTF) in culture medium supplemented with IL-2. After 2 days of activation, the cells were depleted of CD4⁺ T cells. The cytotoxicity of the effector cells was measured in a classic chromium release assay using Na⁵¹CrO₄-labelled TC-1 cells as targets. The percentage of specific lysis was calculated as follows: [(experimental ⁵¹Cr release–spontaneous ⁵¹Cr release)/(maximum ⁵¹Cr release–spontaneous ⁵¹Cr release)×100], where spontaneous release is the radioactivity of the target cells in the absence of effectors (background), and maximum release is the radioactivity detected after treatment of the target cells with Triton X-100.

Chemokine levels in tissues

The levels of chemokines in the tissues (vaginas or TC-1 tumours) at various intervals (8, 24, and 48 h, 4 days, and 7 days) after TLR ligand challenge were measured using

a modified version of the PERFEXT method combined with ELISA [193]. Briefly, mice were sacrificed and vaginas or tumours were excised and weighed before storage at -70°C in PBS that contained phenylmethylsulfonyl fluoride, trypsin inhibitor from soybean, and EDTA. After thawing, the tissue samples were permeabilised with saponin overnight and centrifuged at $16,000 \times g$. Supernatants were analysed for the concentrations of IP-10, MIP-1 α , RANTES, fractalkine, and I-TAC using commercial ELISA kits. The IFN-content was analysed using an in-house ELISA.

Immunohistochemistry

Vaginas or tumours were excised and immediately cryopreserved in embedding medium (O.C.T. Tissue Tek compound). Five-micrometer-thick sections were cut on a cryostat, fixed on glass slides with 100% acetone, and stored at -70°C until use. Endogenous peroxidase activity was blocked by incubating the slides for 20 min in a solution of glucose oxidase, glucose, and NaN_3 . Slides were then incubated with biotinylated anti-mouse I-Ab, H-2Kb, CD4, CD8 or CD11c, followed by avidin–biotin–horseradish peroxidase complex. The sections were then overlaid with the chromogen substrate 3,3-diaminobenzidine, washed with distilled water, counterstained with Mayer's haematoxylin, dehydrated, and mounted with Mountex. Slides without primary antibody were included in all experiments and were consistently found to be negative.

Human studies

Patients

Patients diagnosed with cervical dysplasia by Pap smear were recruited at the time of surgery at the Department of Gynaecology at Sahlgrenska University Hospital, Göteborg, Sweden. We collected heparinised blood samples and immediately used them in the *in vitro* analysis. Vaginal smears were collected and stored at -20°C until analysed for HPV genotypes. Cervical biopsies were sent to the Department of Pathology at Sahlgrenska University Hospital for histopathological diagnosis (normal epithelium, CIN I-IV). Permission for this study was granted by the Ethics Committee of the University of Gothenburg. All the volunteers gave informed written consent.

Healthy volunteers

Buffy coats were obtained from healthy blood donors (C-lab, Blood Supply Unit Sahlgrenska University Hospital, Göteborg, Sweden).

HPV typing

DNA was extracted from vaginal smear samples and used for HPV typing. Both the extraction and typing were performed at the Department of Genetics and Pathology, Uppsala University, Sweden. A modified version of a previously described Real-Time PCR-based assay was used [194], in which three parallel Real-Time PCRs from each DNA sample detect and quantify the amount of the house-keeping gene (HMBS) and the high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 52, and 58. The amplification ramp included an initial hold program of 10 min at 95°C, followed by a two-step cycle consisting of 95°C for 15 s and 57°C for 1 min, which was repeated 40 times. Standard curves ranging from 10^2 to 10^5 copies were established for each HPV type or group of HPV types using plasmids that contained the full genomes of different HPV types. A highly significant linear regression was seen between HPV copy number and threshold cycle (C_t), representing the PCR cycle number in which the signal exceeded a given baseline, for all the HPV types detected by the system. The threshold for a positive HPV type was set at 10 copies per PCR. The results are presented for individual types (16, 31, 35, and 39) and per group (18/45 and 33/52/58).

Monocyte-derived DCs and pulsing with antigen

Monocyte-derived DCs were generated from peripheral blood mononuclear cells (PBMC) prepared from heparinised blood by density grade centrifugation. Monocytes were isolated with CD14+-specific magnetic beads, and DC differentiation was induced by culturing in tissue culture medium that was supplemented with recombinant human GM-CSF and IL-4. After 6 days of culture, immature DCs were obtained. Mature DCs (mDCs) were obtained by adding a cocktail of inflammatory cytokines during the last 24 h of culture. The DCs were harvested by pipetting, washed with PBS, and pulsed for 2 h at 37°C with HPV16-E7 or CT-E7 at a concentration of 0.05 μ M of E7 in culture medium, or with the equivalent (mol:mol) ratio of CT. After pulsing, the DCs were extensively washed to remove any unbound antigen prior to use.

Flow cytometry

Human monocyte-derived DCs were pulsed with 0.02 μ M CT or with medium for 2 h at 37°C, washed, and incubated overnight in 24-well plates. Flow cytometric analyses were performed using directly labelled antibodies to CD14, CD40, CD80, CD83, CD86, HLA-DR, CD11c, and the corresponding isotype controls. The cytometry was performed on a FACS Calibur using the Cell Quest Pro version 5.2.1 software (BD Biosciences). The results were analysed with the FlowJo version 7.2.5 software. Analyses were made on gated DCs that were $\geq 97\%$ CD11c-positive and $\leq 3.5\%$ CD14-positive.

IDO mRNA and kynurenine

IDO mRNA expression was analysed by Real-Time PCR. Total RNA was extracted from 5×10^4 cells. The RNA was treated with DNase to remove any contaminating genomic DNA. cDNA was prepared in a random hexamer-primed Superscript RT reaction according to the manufacturer's protocol. The IDO mRNA levels were quantified by Real-Time PCR in which the primer probe pairs detect and quantify the levels of the house-keeping gene (GAPDH) and IDO. The samples were run in triplicate using the comparative $\Delta\Delta CT$ method of relative quantification to calculate the differences in gene expression between the control and the treated group.

IDO activity was estimated by measuring the levels of kynurenine using high-performance liquid chromatography (HPLC). Briefly, immature DC, CT-DCs, and mature DCs were cultured in cell culture medium with or without recombinant human CD40 ligand and a monoclonal anti-polyhistidine antibody. Supernatants were collected at 24 h and 72 h. HPLC was performed by diluting the supernatants, and kynurenine was detected using a fluorescence detector that was set at an emission wavelength of 480 nm and an excitation wavelength of 365 nm. Quantification was performed with external calibrators of kynurenine, which were run with each assay.

Purification of T cells and the proliferation assay

CD4⁺ and CD8⁺ T cells were obtained from PBMC using specific Dynalbeads, and frozen in liquid nitrogen until used in the proliferation assay. Initial titration experiments of CT-E7-pulsed DCs using E7 concentrations between 0.0005 and 0.5 μM revealed that 0.05 μM of CT-E7 gave a consistent proliferative response without the toxic side-effects of CT. The pulsed and washed DCs were irradiated before they were plated with T cells.

Haemagglutinin and 1-methyl-D-tryptophan

CD4⁺ T cells were plated with immature DCs, CT-pulsed DCs or mature DCs in culture medium for 5 days in the presence or absence of haemagglutinin and 1-methyl-D-tryptophan. Supernatants were collected after 48h and frozen until analysed for IL-12.

Cytokine ELISA

Supernatants from DCs or from co-cultures of antigen-pulsed DCs and CD4⁺ or CD8⁺ T cells were assayed for IFN- γ and IL-12p40 using a specific DuoSet ELISA. IL-13 was analysed using an in-house ELISA.

Statistical analyses

In all the studies, the GraphPad Prism software was used for the statistical analyses. For comparisons of the chemokine and cytokine levels in mice, ANOVA with Dunnett's multiple comparison test was used for comparing more than two groups, while the Student's *t*-test was used for comparing two groups. Differences with *P*-values <0.05 were considered statistically significant.

For the tumour growth analysis, ANOVA with Bonferroni correction for multiple comparisons was used to compare the mean values of the different groups. One-tailed *P*-values <0.05 were considered statistically significant. The frequencies of tumour-free mice were analysed by Fisher's exact test. Two-tailed *P*-values <0.05 were considered statistically significant.

To analyse the cell proliferation and cytokine levels in humans, paired one-way ANOVA (Friedman test) or one-way ANOVA (Kruskal-Wallis test) followed by Dunn's multiple comparison test and the Mann-Whitney test were performed. *P*-values <0.05 were considered statistically significant.

RESULTS

Induction of MHC expression, leukocyte accumulation and chemokine production in the genital tract by TLR ligands

MHC expression and leukocyte infiltration

HPV-infected epithelial cells are difficult to recognize for the immune system since they are located in the outer layers of the genital epithelium and often have a downregulated expression of MHC [116, 195]. Development of treatments that enhance immune responses to HPV-infected cells could include agents that increase the immune recognition of infected/transformed cells in the vaginal epithelium, as well as enhanced recruitment of leukocytes to the infected epithelium. Signalling through TLRs is known to induce the expression of MHC class I and II molecules on many cell types including epithelial cells and DCs [24]. In order to investigate whether TLR signalling would affect the expression of MHC molecules locally in the genital tissues, mice were treated intravaginally with the synthetic TLR ligands poly I:C (TLR3), imiquimod (TLR7) and CpG-ODN (TLR9). Vaginal tissues were collected and stained for MHC class I and MHC class II expression. The MHC class I expression was low in PBS-treated mice and restrained to the basal cells of the vaginal epithelium and a few cells in the lamina propria (Figure 6A and C). Of the different treatments, CpG-signalling via TLR9 had the strongest effects on MHC class I expression (Figure 6B and D). 24h post CpG-ODN treatment, the MHC class I expression was increased both in the basal epithelial membrane and in the cytoplasm of epithelial cells (Figure 6B). Also after 48h there was an increase of MHC class I expression in the epithelium, as well as in the lamina propria (Figure 6D).

There was no detectable expression of MHC class II in the vaginal epithelium of PBS-treated control mice, and only a few cells in the lamina propria and in conjunction with the epithelium were positive for MHC class II. None of the TLR ligand treatments had any profound effect on MHC class II expression in the vaginal epithelium (data not shown). CpG treatment induced an accumulation of leukocytes within the lamina propria (Figure 6D). These infiltrating cells were found to be both MHC class II positive (Figure 7B) and CD11c positive (Figure 7D) which indicate that they were DCs.

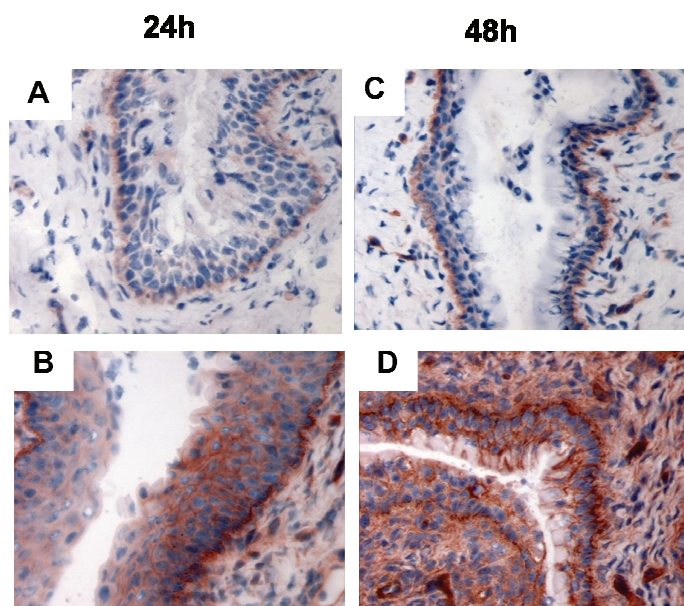


Figure 6. MHC class I expression in vaginal tissue after local treatment with CpG-ODN. Frozen sections of vaginal tissue was stained for MHC class I expression 24 h (A and B) and 48 h (C and D) after administration of PBS (A and C) or CpG-ODN (B and D).

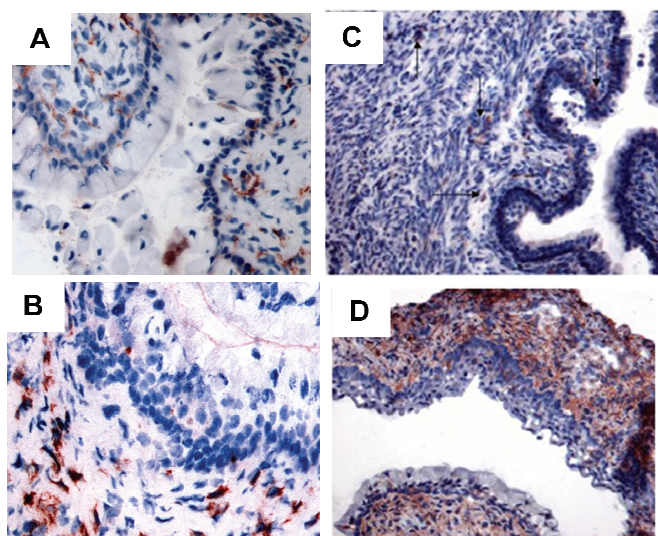


Figure 7. MHC class II and CD11c expression in vaginal tissue after local treatment with CpG-ODN. Frozen sections of vaginal tissues were stained for MHC class II expression (A-B) and CD11c expression (C-D) 48h after intravaginal administration of PBS (A and C) and CpG-ODN (B and D).

Chemokine production

TLR-signalling is known to induce the production of chemokines and the infiltration of effector cells into tissues. We measured the intravaginal chemokine levels of IP-10 (CXCL10), MIP-1 α (CCL3), RANTES (CCL5), fractalkine (CX₃CL1), and I-TAC (CXCL11) after intravaginal administration of poly I:C, imiquimod or CpG-ODN. All treatments induced high but transient levels of IP-10 eight hours after treatment (Figure 8A). Imiquimod and CpG-ODN also induced MIP-1 α . The strongest effect was seen after treatment with CpG-ODN which induced high levels already after 8h post treatment, and these levels were sustained for up to 7 days (Figure 8B). Production of RANTES was strongly upregulated by CpG-ODN 24h after treatment but had disappeared after 48h, while poly I:C induced a peak in RANTES production after 8h (Figure 8C).

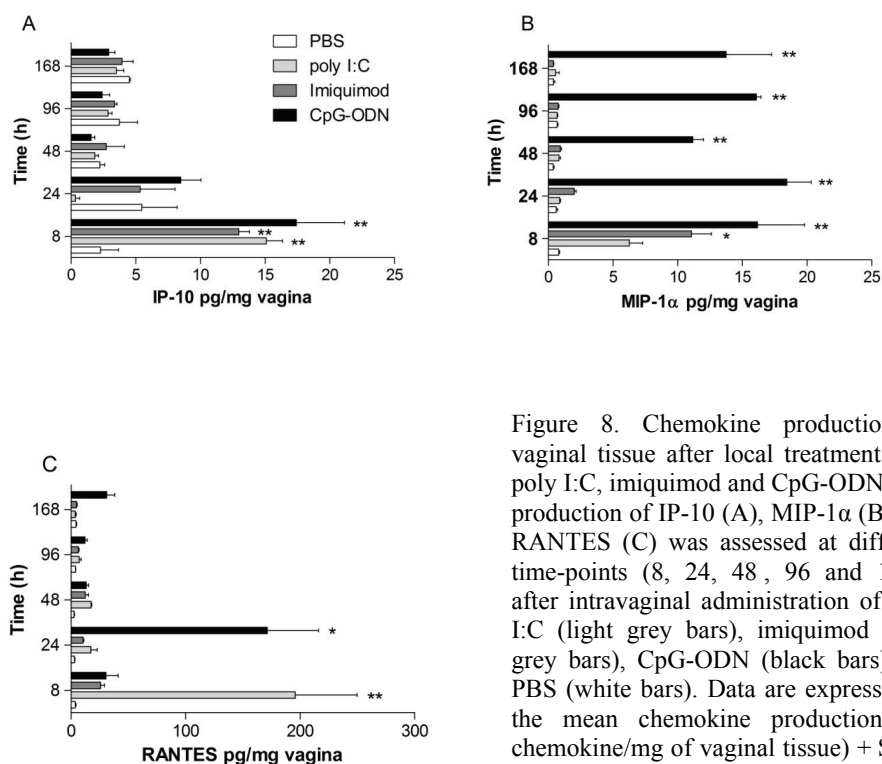


Figure 8. Chemokine production in vaginal tissue after local treatment with poly I:C, imiquimod and CpG-ODN. The production of IP-10 (A), MIP-1 α (B) and RANTES (C) was assessed at different time-points (8, 24, 48, 96 and 168h) after intravaginal administration of poly I:C (light grey bars), imiquimod (dark grey bars), CpG-ODN (black bars) and PBS (white bars). Data are expressed as the mean chemokine production (pg chemokine/mg of vaginal tissue) + SEM, with three to six animals per group. * = $p < 0.05$, ** = $p < 0.01$.

Induction of MHC expression, leukocyte accumulation and chemokine production in experimental tumours by TLR ligands

MHC expression and leukocyte accumulation

To assess if CpG-ODN could induce MHC class I and II expression also in tumours and thus increase the potential recognition by T cells, we injected CpG-ODN into localised intradermal TC-1 tumours. The TC-1 tumours cells express HPV16 E6 and E7 and are injected intradermally where they form an encapsulated and rapidly growing tumour. Indeed, intratumoural injection of CpG-ODN increased the number of MHC class I and II expressing cells in the tumour 10 days after tumour inoculation (Figure 9).

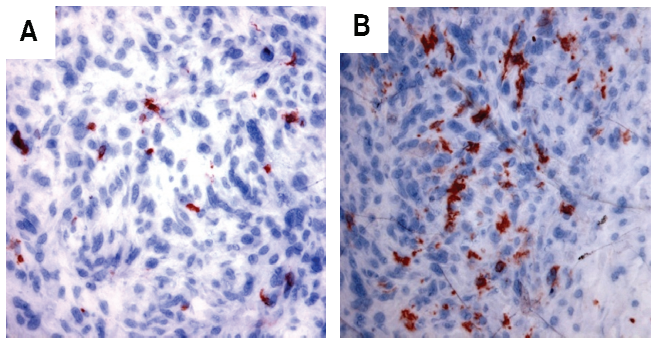


Figure 9. MHC class II expression in tumours after intratumoural treatment with CpG-ODN. Mice were injected with TC-1 tumour cells, and treated with PBS (A) or CpG-ODN (B) at day 2 and 8 after TC-1 injection. Ten days after TC-1 injection, tumours were collected, frozen and stained for the expression of MHC class II (I-A^b).

Chemokine production

Induction of robust anti-tumour responses correlates with tumour T-cell infiltration. Treatments that enhance chemokine expression within a tumour might promote trafficking of T cells to the tumour, and thereby facilitate tumour eradication. We investigated if intratumoural administration of CpG-ODN could enhance the chemokine production in TC-1 tumours. Levels of IP-10, MIP-1 α and RANTES were measured in the tumours 8h and 24h after CpG-ODN injection. MIP-1 α and RANTES were induced at 8h and sustained at 24h, while IP-10 could not be detected at any time point (Figure 10).

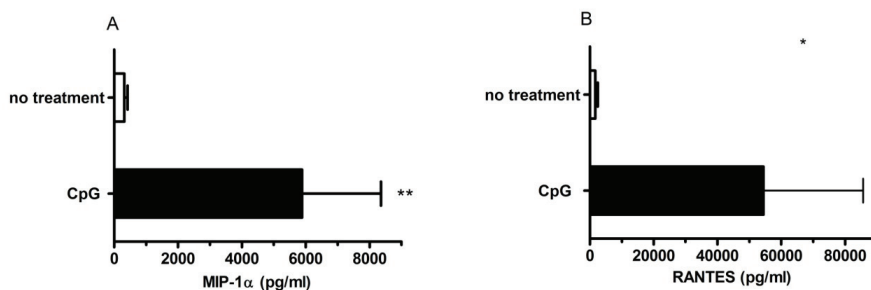


Figure 10. Chemokine production in TC-1 tumours after local treatment with CpG-ODN. The production of MIP-1 α (A) and RANTES (B) was assessed 8h after injection of CpG-ODN (black bars) and PBS (white bars). Data are expressed as the mean chemokine production (pg/ml) \pm S.D. of four animals per group. * = $p < 0.05$, ** = $p < 0.01$.

CT as a combinational carrier/adjuvant promotes DC vaccination efficiency against an experimental TC-1 tumour *in vivo*

CT-E7-DC vaccination is associated with reduced tumour growth and the priming of tumour-specific CTLs

We have earlier reported that pulsing of DCs with CT-conjugated antigens augments the antigen-presenting capacity of DCs leading to enhanced antigen-specific T-cell responses. The concept of using CT-conjugated antigens to enhance T-cell responses was tested in an ovalbumin (OVA)-tumour model, where vaccination with OVA-pulsed DCs eradicated growth of OVA-expressing tumours [188, 189]. We now wanted to assess if DC vaccination with CT-conjugated antigen also would have the same beneficial effects with a tumour expressing the real oncoproteins HPV16 E6 and E7. Mice were injected subcutaneously with the tumour cell line TC-1 and then vaccinated three times at weekly intervals. Vaccination with CT-E7-pulsed DCs, but not E7-pulsed DCs, resulted in a significantly slower tumour growth compared to untreated controls (Figure 11). Vaccination with CT-E7-DCs induced tumour clearance in 11% of the mice. No tumour eradication was seen in mice vaccinated with E7-pulsed-DCs or CT-pulsed DCs. Thus, CT-E7-pulsed DCs conferred an advantage over E7-pulsed DCs for the reduction in tumour burden.

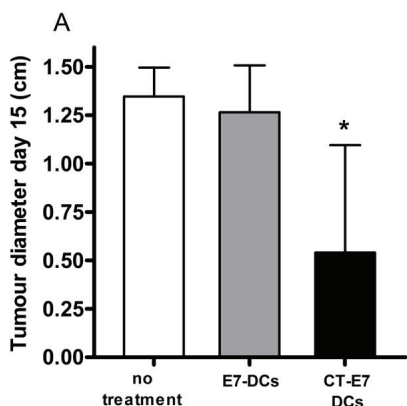


Figure 11. TC-1 tumour size after vaccination with CT-E7-pulsed DCs or E7-pulsed DCs. Mice were injected with TC-1 cells and then treated with PBS (white bar), E7-DCs (grey bar) or CD-E7-DCs (black bar) two times at weekly intervals, starting two days after the TC-1 injection. CT-E7-pulsed DCs (black bar) but not E7-pulsed DCs (grey bar) significantly reduces tumour size ($n = 4$). One representative experiment out of two is shown. All data are shown as tumour diameter \pm S.D. * $P < 0.05$.

Clearance of tumours is dependent on efficient CTL responses. We therefore wanted to investigate if vaccination with CT-E7-pulsed DCs could activate CTL effector cells. For this purpose, mice were vaccinated twice with CT-E7-pulsed DCs. Two weeks after the second vaccination, spleen cells were harvested and activated with a CD8+ specific HPV16 E7 peptide *in vitro*. Enriched CD8+ T cells were then assayed for cytotoxicity against the target cells, TC-1. Activated CD8+ T cells from CT-E7-DC vaccinated mice were significantly more potent tumour-specific CTLs (Figure 12).

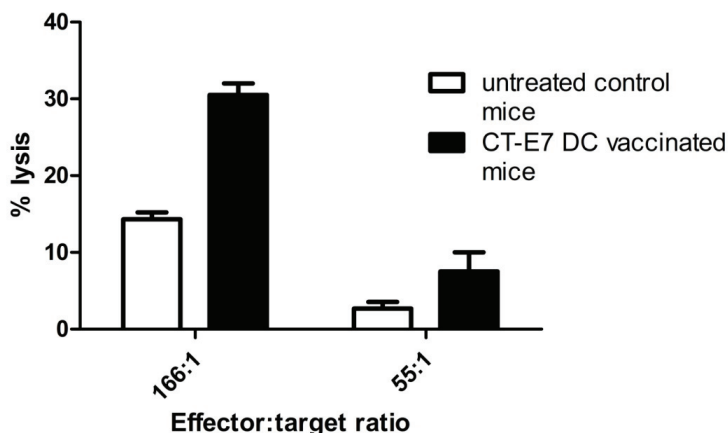


Figure 12. Vaccination with CT-E7-pulsed DCs is associated with E7-specific CTL responses. Data show lysis of TC-1 ^{51}Cr -labelled target cells by purified CD8+ T cells effector cells in untreated mice (white bars) and CT-E7-DC vaccinated mice (black bars). Data are expressed as mean percentage of lysis \pm S.D.

Tumour eradication using a combinational therapy (CT-E7-pulsed DCs + CpG-ODN)

Since signalling through TLRs has been reported to reduce tumour growth *per se*, we wanted to assess if the anti-tumour effects seen with CT-E7-DC vaccination could be enhanced by combining the vaccine with intratumoural injection of the TLR ligands poly I:C, imiquimod or CpG-ODN. The combination of CT-E7-pulsed DCs and CpG-ODN was by far the most effective treatment leading to complete tumour eradication in all treated animals (Figure 13).

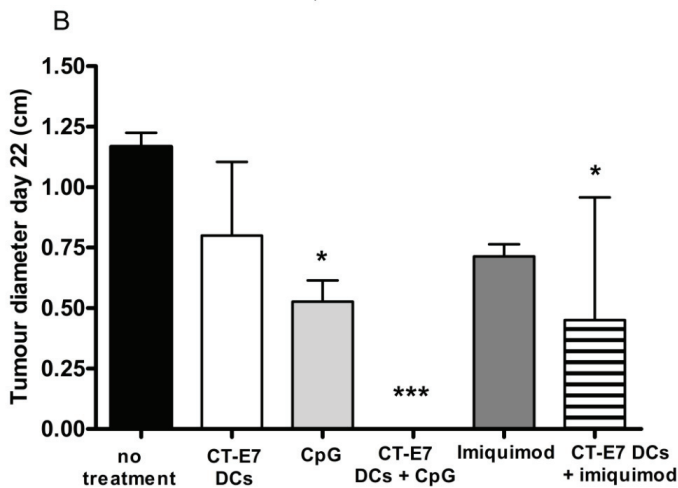


Figure 13. TC-1 tumour size after therapeutic vaccination with CT-E7-pulsed DCs, or TLR ligands or combinational treatment. Mice were injected with TC-1 cells and then treated with CT-E7-DCs, intratumoural CpG-ODN or imiquimod, or combinations three times at weekly intervals, starting two days after the TC-1 injection. All data are shown as tumour diameter \pm S.D. * $P < 0.05$, *** $P < 0.001$.

Tumour rejection following DC vaccination requires CD8+ T cells

The TC-1 tumour model has been reported to depend on CD8+ T cells for anti-tumour immunity [196]. We therefore examined if tumour eradication following combinational treatment was dependent on CD8+ T cells. TC-1 cells were injected in CD8^{-/-} mice and then mice were treated with CT-E7-pulsed DCs in combination with CpG. CTLs were indispensable since mice lacking CD8+ T cells could not eradicate their tumours (Figure 14).

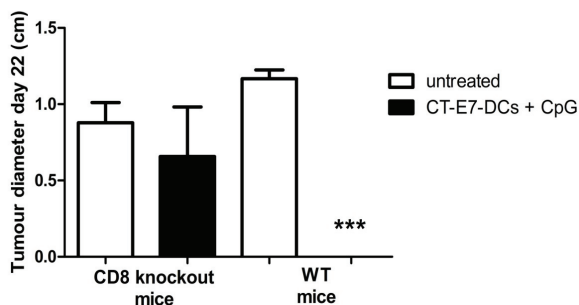


Figure 14. Tumour eradication in response to CT-E7-pulsed DCs and CpG in WT and CD8^{-/-} mice. Mice were injected with TC-1 cells and then treated with PBS or CT-E7-pulsed DCs and intratumoural CpG-ODN three times at weekly intervals, starting two days after the TC-1 injection. All data are shown as tumour diameter \pm S.D.

Conjugation of HPV16 E7 to cholera toxin enhances the HPV-specific human T-cell recall responses to pulsed DCs *in vitro*

Characterisation of study population

The HPV genotype distribution

The HPV types infecting the 35 individuals included in the study were assessed by RT-PCR on DNA from vaginal smears. We tested some of the most common oncogenic HPV types (HPV16, HPV31, HPV35, HPV39, HPV18/45 and HPV33/52/58). The most common HPV type was HPV16 infecting 16 of the patients (Table 8). Multiple infections were found in at least nine of the 35 patients (26%). Ten of the patients were negative for HPV virus meaning that they had either cleared the virus or that they were infected with a less oncogenic HPV type (Table 8).

Table 8. The HPV-infection status in the cohort

<i>HPV-type</i>	<i>Number of patients</i>	<i>Percent</i>
16	16	46
18/45	6	17
33/52/58	10	29
31	4	11
35	1	3
39	0	0
Other/negative	10	29

Histopathological status

Cervical tissue specimens obtained at surgery (i.e. 1-3 months after a positive Pap smear) were used for histopathological diagnosis of the 35 patients (Table 9). The patients were diagnosed with normal epithelium, CIN I (mild intraepithelial neoplasia), CIN II (moderate intraepithelial neoplasia) , CIN III (severe intraepithelial neoplasia or carcinoma *in situ*) and CIN IV (cervical cancer). 75% of the patients with normal cytological status had no detectable levels of viral DNA. One patient who had HPV16 and one patient with at least three ongoing HPV infections, had reversed to normal cytology. HPV16 was more common among patients with CIN II-IV (57%) than among patients with normal cytology/CIN I (29%).

Table 9. Histopathological data of the cohort

<i>Histology</i>	<i>Number of patients</i>	<i>Percent</i>
normal	8	23
CIN I	6	17
CIN II	10	29
CIN III	10	29
CIN IV	1	3

CT-conjugation of E7 promotes the HPV-specific T-cell recall responses to pulsed DCs

Based on our findings that DC vaccination with CT-conjugated E7 led to strong anti-tumour responses in mice, we wanted to assess if this was true also in humans. Monocyte-derived DCs from patients with HPV-induced cervical disease were therefore pulsed with CT-E7 and co-cultured with autologous T cells. Since a robust Th1 response is necessary for *in vivo* tumour eradication, we also measured the production of IL-12 and IFN- γ in these cell cultures. The T-cell responses to E7 pulsed DCs were weak or negligible. CT-conjugation of E7 greatly improved the capacity of antigen-pulsed DCs to activate T cells. The CD4⁺ T cells responded to CT-E7-pulsed DCs with a strong proliferation combined with a modest production of IFN- γ (Figure 15A-B). The responses were antigen-specific, since CT-pulsed DCs could not evoke proliferative responses. CT-E7-pulsed DCs co-cultured with CD4⁺-or CD8⁺ T cells produced significantly more IL-12, as compared to E7-pulsed DCs (Figure 15C).

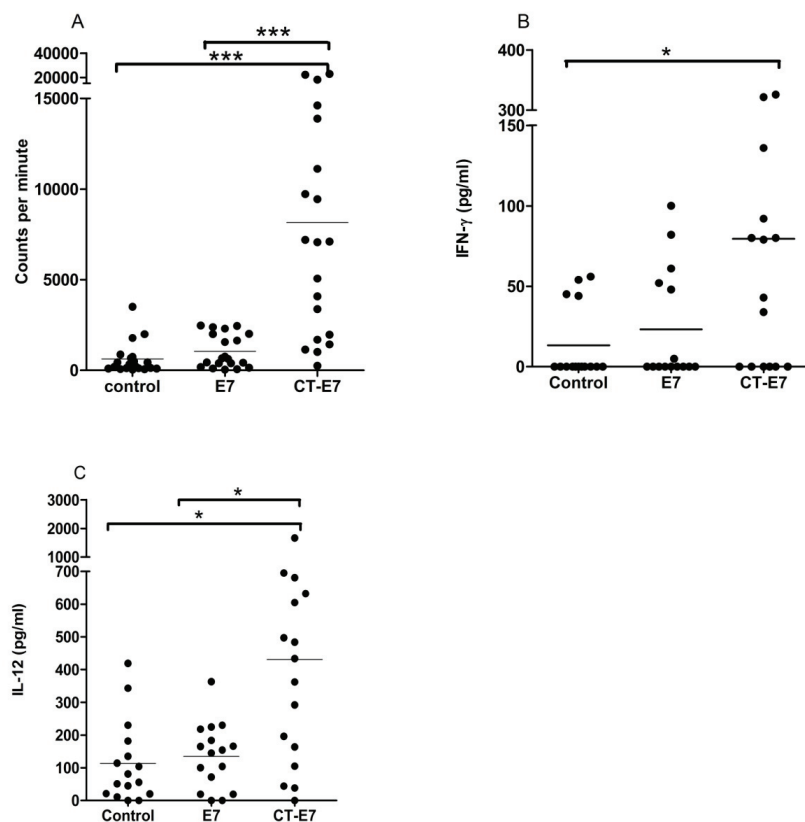


Figure 15. E7-specific T-cell recall responses to DCs pulsed with CT-conjugated HPV16 E7. Unpulsed, HPV16 E7-pulsed and CT-E7-pulsed immature DCs from women with cervical dysplasia were incubated with autologous CD4⁺ T cells for 6 days. Proliferative responses (A), IFN- γ production (B), and IL-12 production (C) are shown. Data are presented as individual values with mean values for proliferation (cpm) and cytokine production (pg/ml). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using Friedman test followed by Dunn's multiple comparison test for $n=15-20$.

Using CT-E7-pulsed DCs, it was possible to induce a CD4⁺ T-cell proliferative response both in patients with normal cytology and in patients with different grades of cervical disease (Figure 16A). Interestingly, CD4⁺ T cells from patients with normal cytology produced more IFN- γ as compared to patients with different grades of CIN, but the differences were not significant due to the limited number of patients (Figure 16B).

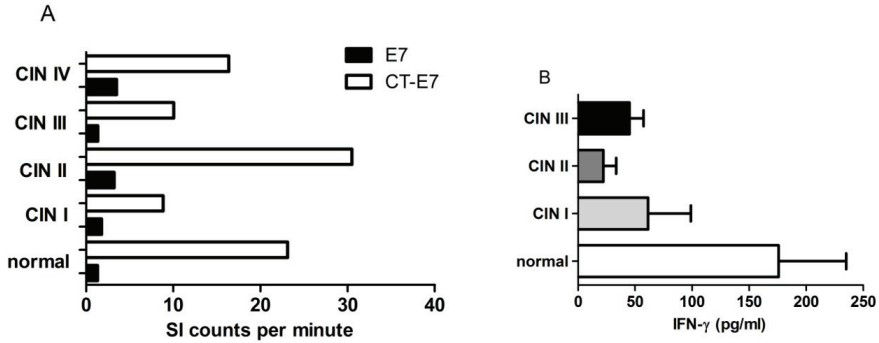


Figure 16. E7-specific T-cell recall responses to DCs pulsed with CT-conjugated HPV16 E7. HPV16 E7- and CT-E7-pulsed DCs from women with different grades of cervical dysplasia were incubated with autologous CD4⁺ T cells for 6 days. Proliferative responses (A) and IFN- γ production (B) are shown. The cohort was divided into patients with normal cytology, CIN I, CIN II, CIN III and CIN IV. Data are presented as mean proliferation values (cpm) and the means \pm SEM for IFN- γ production (pg/ml) using Kruskal-Wallis test followed by Dunn's multiple comparison test for $n=4-9$.

IDO expression in CT-treated DCs

IDO transcription and enzymatic activity in CT-treated DCs

The enzyme indoleamine 2,3-dioxygenase (IDO) has an immunoregulatory role and has been proposed to negatively affect the success of therapeutic DC vaccination against cancer [46]. We investigated if CT would affect IDO production by human DCs. CT-pulsed DCs were compared to DCs treated with maturation cytokines and the expression of IDO mRNA was measured. CT-pulsed DCs showed a significantly lower expression of IDO mRNA compared to mature DCs (Figure 17A).

To determine whether the expression of IDO mRNA resulted in the production of functionally active IDO protein, the activity of IDO was detected by measuring the accumulation of the tryptophan metabolite kynurenine. Kynurenine is produced by the degradation of tryptophan by IDO to N-formyl-kynurenine, which is rapidly converted to kynurenine. The IDO activity was low in CT-DCs at 24h compared to the mature DCs. No IDO activity was present in immature control DCs (Figure 17B). Hence, CT-pulsing of immature DCs does not induce IDO production.

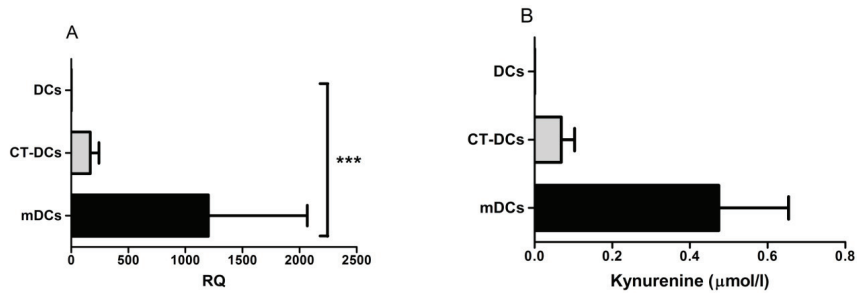


Figure 17. IDO mRNA expression and IDO activity in human DCs. Immature DCs were left either untreated, CT-pulsed for 2 hours, or treated for 24h with maturation cocktail (PGE2, TNF- α and IL-1 β). (A) IDO mRNA was analysed by RT-PCR. (Relative quantification was calculated by IDO/GAPDH ratios. Untreated cells were used as calibrator sample and set to a value of 1.0. Data are expressed as mean \pm SEM ($n=11$). * $P < 0.05$. (B) IDO activity. Kynurenine levels of concentration were measured at 24h. Data are expressed as means \pm SEM ($n=3$) experiments, * $P < 0.05$.

IDO expression in CT-primed DCs after CD40 ligand activation

To investigate if CT would prime DCs for later activation by T cells, we stimulated DCs with CD40L. Twenty-four hours later, the cells and the supernatants were screened for IDO mRNA and IDO activity as above. Stimulation with CD40L induced high expression of IDO mRNA in CT-pulsed DCs (Figure 18A). Also the kynurenine levels were high in CT-pulsed DCs and thus correlated with the mRNA expression (Figure 18B).

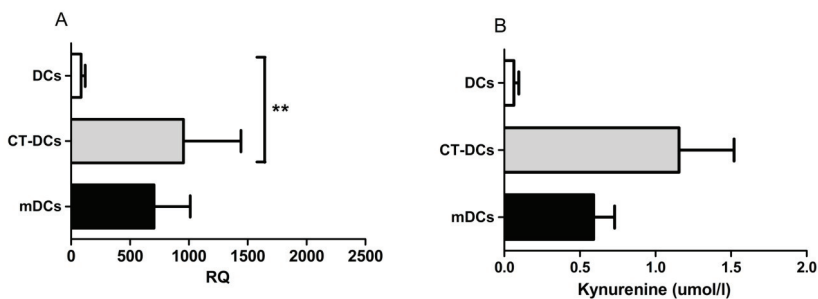


Figure 18. Effects of anti-CD40 Ligation on IDO mRNA levels and IDO activity in human DCs. Immature DCs, CT-DCs and mature DCs were stimulated with anti-CD40L for 24h, 48h and 72h. (A) IDO mRNA expression was measured after 24h of anti-CD40L treatment and analysed by RT-PCR. Relative quantifications were calculated by IDO/GAPDH ratios in each sample ($n=6$). Untreated cells were used as calibrator sample and were set to a value of 1.0. Data is shown as means \pm SEM. ** $P < 0.05$. (B) IDO activity after 24h treatment ($n=3$).

DISCUSSION

Dendritic cells play an important role in the initiation and regulation of tumour-specific immune responses *in vivo* and these cells have therefore the potential to be used as therapeutic cancer vaccines. The concept of DC vaccination is that DCs could overcome tumour immune tolerance and instead enhance the anti-tumour T-cell responses. In the ideal situation, *in vitro* manipulated DCs should be able to elicit anti-tumour immunity *in vivo* upon administration to cancer patients [150]. However there is still a big gap between the ideal scenario and the clinical reality, and so far therapeutic DC vaccinations have not been successful in treating cancer patients [197]. Some recent promising DC vaccination studies in melanoma patients [198] and hormone-refractory prostate cancer [163] have however been reported. There have only been few clinical therapeutic DC vaccination trials against HPV-induced cancer [147, 151, 152, 199]. Even though the immunological responses have been demonstrated in some patients, there have been no objective clinical results. Unfortunately, patients in clinical trials are often unable to respond to immune-stimulating vaccines because of the immunosuppression caused both by earlier treatments and by their heavy tumour burden [170]. In addition, the choice of method to activate the DCs in order to achieve optimal anti-cancer immunity in patients is a controversial topic, and several methods exist to generate mature DCs [150, 158]. Other parameters that are critical for successful DC vaccination include the source of DCs [22], the choice of antigen and method of antigen loading [200] and the route of administration [159]. We believe that the use of CT-conjugated antigen has several advantages for therapeutic DC vaccination. Firstly, CT potentiates antigen uptake and presentation on both MHC class I and MHC class II [177, 189, 201]. Secondly, CT activates the DCs and leads to enhanced IL-12 production ([180, 181] and paper III), which is a requirement for CTL induction. Thirdly, CT does not *per se* induce expression of the immunoregulatory enzyme IDO in exposed DCs (paper IV).

Tumour cells have a variety of mechanisms to evade detection by immune cells which represents a major obstacle when trying to induce anti-tumour immunity in cancer patients. Tumour cells often interfere with antigen-presentation and downregulate the expression of surface MHC-tumour antigen complexes leading to escape from recognition by cytotoxic CD8⁺ T cells, the main eliminators of virus-infected cells and tumour cells [202, 203]. This is indeed true also for HPV; HPV E7 is interfering with the TAP molecule thereby decreasing surface expression of MHC class I [121, 204]. The loss of MHC class I is associated with the pathogenesis of HPV infection and found in both neoplasia [205] and cervical cancer [122]. Tumours also create a non-inflammatory and immunosuppressive milieu which facilitates the escape from

the immune system. The productive life-cycle of an HPV-infection is non-lytic. Thus, there is no cell death and subsequent inflammation. Thereby, the danger signals which would normally initiate Langerhans cell maturation and migration to lymph nodes are avoided, and no T-cell activation is achieved [78, 206, 207]. The immunosuppressive mechanisms could also be a consequence of, e.g. tumour cell production of IL-10 [208] and TGF- β [209]. Some of these responses are skewing the immune responses towards a Th2 profile which is less effective than a Th1 response in anti-tumour defence [118]. Other mechanisms include the inhibition of DC activation and function [210], and defects in T-cell activation and function [118, 211, 212]. Defects in the activation of the innate immunity have also been reported [213].

One way to abrogate the invisibility of HPV-infected epithelium and HPV-induced tumours could be to enhance the expression of MHC on epithelial cell surfaces and on DCs present in the tissues. Thus, the number of epitopes being presented to both CD4⁺ and CD8⁺ T cells would then be increased and the virus-transformed cells would be easier targets for immune-mediated destruction [11]. TLR-signalling by CpG has earlier been reported to induce *in vivo* expression of MHC molecules by tumour cells [214], and this was confirmed by us in paper II. We could also show that the use of poly I:C, imiquimod and CpG as intravaginal treatments in mice did induce MHC expression in the vaginal tissues, with different kinetic patterns for the different TLR ligands. CpG treatment stimulated the most pronounced and prolonged expression of MHC class I both in the vaginal epithelium and lamina propria, and also induced increased numbers of cells in the lamina propria with strong expression of MHC class II. CpG administration intratumourally also enhanced the expression of MHC class I and II on TC-1 tumours *in vivo*. Thus, we propose that CpG could be used to overcome the HPV-induced downregulation of MHC on infected cells also in humans.

Abrogation of the anti-inflammatory and immunosuppressive milieu caused by HPV could also be achieved by enhancing signalling through TLRs. TLR ligation cause inflammatory responses with chemokine production, which is crucial for a rapid influx of leukocytes into inflamed tissue [215]. By the administration of well-defined TLR ligands one could control which chemokines that were induced and thus which leukocytes that were attracted. We could demonstrate that intravaginal administration of ligands for TLR3, 7 and 9 enhanced the production of IP-10, MIP-1 α and RANTES in the vagina. These chemokines are attractors of CD4⁺ Th1 cells, cytotoxic CD8⁺ T cells and NK cells and MIP-1 α of DCs [216-218]. The chemokine production induced was associated with an enhanced infiltration of cells to the lamina propria. After CpG treatment, these cells were shown to be CD11c-positive DCs, which were most likely responding to the strong and sustained production of MIP-1 α induced following

treatment CpG administration also enhanced the production of MIP-1 α and RANTES in TC-1 tumours, which was associated with an influx of MHC class I and II positive cells into the tumour.

We further analysed the CpG-treated vaginal tissues and tumours for presence of CD4⁺ Th1 cells and CTLs, i.e. cells that are important for the elimination of virus-infected cells and tumour cells. These cells express receptors for IP-10, MIP-1 α and RANTES and we therefore hypothesised that they should respond to the chemokines induced and migrate into the tissue. We could however not document any T-cell infiltration to the vaginal tissues when analysed 48h post treatment. This means either that we choose the wrong time-point for analysis, or that the chemokines induced by the CpG treatment are not the main attractors of these cells into the genital tract. We did find a few CD4⁺ and CD8⁺ T cells in CpG-treated tumours. These were analysed one day after the second administration of CpG, i.e. just before the tumours started to decrease in size in successfully treated animals. Given the relatively rapid decline in tumour size after this time-point, it was difficult to obtain big enough pieces of tumour for analysis. However, we envision that the numbers of T cells would increase concomitantly with the immune-mediated destruction of the tumour.

Intravaginal treatment with CpG was more efficient than poly I:C and imiquimod in inducing in the genital tract the expression of MHC molecules, the production of chemokines, and the recruitment of leukocytes, at least in mice. It needs to be established if CpG could be used to enhance MHC expression and leukocyte recruitment also in humans with HPV-induced cervical disease, or if any other TLR ligand would be more efficient in the human system. In humans, TLR ligands have become interesting targets for cancer immunotherapy, either as treatments *per se* or as adjuvants. Focus has been on CpG (reviewed in [219]) and ligands for TLR7 and 8 (reviewed in [220]). Clinical trials using TLR agonists as enhancers of the anti-tumour response in solid tumours have begun (reviewed in [221]). Imiquimod 5% cream is already used as treatment for HPV-induced genital warts [220] and to treat malignant tumours of the skin. Intratumoural injection of agonists for TLR2 and 6 has been used in patients with pancreas carcinomas [222]. The effect of intratumoural CpG administration on HPV-induced cancers needs to be established. Furthermore, we do not know if treating tumours with CpG could counteract the reported downregulation of TLR9 by HPV16 [117], or if signalling through one of the other TLRs would be more efficient. One way to clarify this could for instance be to administer different TLR ligands to surgically removed genital tissues from patients with cervical dysplasia, and then analyse the biopsy for chemokine production and MHC expression.

CT is an efficient antigen carrier and adjuvant for DC vaccination. Even though CT is toxic *per se*, it was well tolerated in mice when used for DC pulsing. When CT-conjugated antigen is used in DC vaccination, it has strong effects on both the antigen uptake/presentation and the activation/maturation of DCs. The CTB subunits, which bind to the GM-1 ganglioside, greatly enhances both antigen uptake and presentation [182]. Inclusion of the biologically active A subunit further increases the T-cell activating capacity through its ADP-ribosyltransferase activity that increases intracellular cyclic APM levels [188, 201]. Studies from our research group show that mice carrying ovalbumin-expressing tumours can clear the tumour after therapeutic vaccination with CT-ovalbumin-pulsed DCs [188, 189]. The CT treatment induced enhanced levels of costimulatory molecules and MHC class I on DCs, which led to the activation of highly cytotoxic IFN- γ secreting CD8⁺ T cells in vaccinated animals. However, in the TC-1 tumour model used in my work, vaccination with CT-conjugated E7 was not enough for tumour eradication. There could be numerous reasons for this discrepancy, e.g. the difference in tumour cell origin and MHC composition, and the nature of the antigen that was transfected into these tumour cell lines. The EG.7 tumour cells used in the OVA-model derive from a murine T-cell lymphoma and probably express more MHC than the TC-1 cells which are of epithelial tumour origin. If so, the EG.7 cells could be better targets for CTLs. Also, HPV16 E6 and E7 which are present in the TC-1 cells are real tumour oncogenes and could affect many processes in the cells, e.g. antigen presentation, susceptibility to CTL-induced apoptosis, as well as general effects on the cell cycle. It could also be that the E7 used for DC pulsing had a negative effect on the antigen-presenting and T-cell activating capacity of these DCs.

CT-conjugation of E7 was necessary for the generation of an efficient DC therapeutic vaccine against TC-1 tumours in mice. The combination of CT-E7-pulsed DCs with intratumoural CpG did induce the eradication of developing tumours, while DCs pulsed with E7 alone was not sufficient, irrespective of combinational treatments. The successful therapeutic treatment induced IFN- γ secreting CTLs which were proven essential for decreasing the tumour burden [187]. One unsolved issue is of course whether CT-pulsed DCs can be used in humans. To our knowledge, CT has never been tested *in vivo* as an adjuvant due to its toxic effects. However, if used in a DC vaccine, one could refine the handling procedure, in particular the post-pulsing washing, to ascertain that no free or unprocessed CT was left in the mixture upon injection.

Cellular immunity, particularly CD4⁺ T-cell activation, is crucial to control HPV infections. Occurrence of HPV-specific CD4⁺ T cells in the blood is associated with disease regression, both in women with different grades of CIN and in those with

cervical cancer [103, 119, 146]. The complex role of CD4⁺ T cells in anti-tumour immunity (reviewed in [141]) involves the recruitment of macrophages, granulocytes, and NK cells to the tumour site [11, 12], the activation of tumour-specific CTLs [11, 12], and the functional assistance of NK-cells [223], i.e. cells which are important in anti-tumour defence. Activated CD4⁺ T cells also provide help to DCs through the CD40 ligand whereby the production of the key Th1-inducing cytokine IL-12 is induced in the responding DCs. IL-12 initiates the Th1 responses which are crucial for anti-tumour immunity [6-9]. The main Th1 cytokine is IFN- γ , and immune responses against HPV-induced cervical disease are dependent on IFN- γ production. This has been demonstrated in both precancerous lesions and in cervical cancer [224-226]. Regression of HPV infection is also associated with increased IFN- γ production, while defective IFN- γ production may be correlated with persistent HPV infections and progression of disease. IFN- γ production by Th1-cells enhances the cytotoxicity of CTLs and macrophages [13, 141], and upregulates MHC on tumour cells making them easier targets for effector cells [13, 141]. In my *in vitro* study of human recall responses to HPV E7, I could show that CT-E7-DCs induced strong E7-specific proliferative responses in CD4⁺ T-cells from women with different grades of cervical disease. These responses were characterised by high levels of IL-12 and moderate levels of IFN- γ . Even though the E7-specific IFN- γ responses were a bit low and therefore somewhat discouraging, it should be noted that they were far superior to those obtained in other studies where elaborate *in vitro* re-stimulations and expansions of the activated T-cell clones had to be performed before obtaining any measurable E7-specific T-cell responses [147].

Cytotoxic CD8⁺ T cells are crucial in anti-tumour immunity. In cervical cancer patients, a high number of intraepithelial CD8⁺ T cells infiltrating the tumour is associated with a better prognosis [227]. However, CD8⁺ T cells appear to be difficult to activate *in vitro*. In our *in vivo* model of therapeutic DC vaccination in mice we got relatively poor *in vitro* CTL responses even though we could clearly show that the CD8⁺ T cells were fundamental for tumour clearance. In the human set-up, the CT-E7-pulsed DCs did not induce any CD8⁺ T-cell recall responses. However, we suggest that the E7-specific Th1-dominated responses achieved *in vitro* could give activation of CD8⁺ T cells *in vivo*, similarly as demonstrated in the mouse model.

It would be interesting to investigate whether the CD8⁺ T cells activated with CT-E7-pulsed DCs become potent CTLs. To perform *in vitro* CTL assay on cells from cancer patients, cells from tumour tissue are often used as target cells [228, 229]. We did not have access to cervical tissue from the patients and could therefore not perform such assays. Instead, I spent one year trying to express HPV16 E6, E7 and L1 in eukaryotic cells. The purpose was two-fold. Firstly, I wanted to produce proteins with an

eukaryotic methylation pattern to use as vaccine. Secondly, I wanted to use the vectors to transfect cells from the patients and then use them as targets in cytotoxicity assays. However, even though I did manage to insert the appropriate sequences into plasmid vectors and express these in bacteria, I was not able to express the proteins in eukaryotic cells. Successful transfection of cells inevitably led to cell death, perhaps as a consequence of the cell-cycle-modifying capacities of these proteins. Another alternative which we have never pursued was to pulse potential target cells with immunodominant HPV E7 CTL epitopes. Such a procedure would have required a prior HLA typing of the patients, and this was unfortunately not performed.

Papillomaviruses are strictly species-specific which makes it difficult to study human papillomaviruses in animal models. Therefore, animals infected with animal papillomaviruses have been widely used to study both prophylactic and therapeutic vaccinations [230]. None of the animal papillomaviruses are however optimal for studies of genital HPV; either the lesions are not in the genital mucosa, the virus is not malignant, or the virus has a completely different life cycle [230] [231]. There is no species-specific rodent papillomavirus known that could be used in small laboratory rodents. There are however several rodent models in use. One way to study HPV growth and propagation *in vivo* has been to use immunocompromised mice and transplant them with human tissue xenografts infected with different HPV types [232, 233]. Another transplantation model is used to study the immune responses to E6 and E7; skin grafts from transgenic mice that express HPV E6 and/or E7 from an epithelial-restricted promoter are transplanted into immunocompetent mice. Studies performed in this model show that E6 and E7 evoke very weak immune responses which do not lead to graft rejection [234]. A very interesting new mouse model with several advantages is the *K14E7* transgenic mouse strain. This strain of mice express HPV16 E6 and/or E7 in stratified squamous epithelia, i.e. the natural sites for HPV16 infection, and the animals develop cervical cancer upon oestrogen treatment [235]. This model is reminiscent of human cervical malignancy as it progresses with several different stages of cervical carcinogenesis and is located in the cervix. There is also a range of mouse transplantable tumours that express HPV proteins. One of these is the TC-1 epithelial tumour cell line used by me in my studies, and which expresses the main viral oncogenes E6 and E7.

There are limitations with my TC-1 model. The tumour grows in the skin epithelium and not in the cervix, mainly due to technical issues. Another limit is the early time point we chose for vaccination. In humans, tumours often are discovered at a later time-point which perhaps makes them more difficult to treat; in cancer, an early treatment is more likely to be successful, and this is true both in mice and in humans. Another limit with our model is that the TC-1 cells, when growing in the skin, do not

metastasise and thereby differ from real cervical cancer. Taking all this into account, there are also advantages with the TC-1 mouse model, in particular the fact that the *in vivo* immune responses obtained in mice are similar to those achieved *in vitro* in the human system.

The TC-1 tumour model has been used to test the effects of several different therapeutic treatments. The most successful treatment that has been reported consisted of an immunodominant MHC class I-restricted E7 peptide given with 4-1BBL as an adjuvant [236]. 4-1BB is a costimulatory molecule which is a member of the tumour necrosis factor receptor family. It is expressed on activated CD4+ and CD8+ T cells, and stimulation with its ligand 4-1BBL (provided by activated APC) leads to T-cell activation, clonal expansion, survival and long-term memory [237]. In this protocol the soluble 4-1BBL was fused to the COOH terminus of a modified core streptavidin which enables the chimeric SA-4-1BBL to exist as tetramers and oligomers. This leads to cross-linking of the 4-1BB receptors on T cells which potentiates the signal. This prototype vaccine induced complete eradication of 10-day established tumour in 75% of the mice after only one vaccination and led to long-term survival. In our studies the mice were vaccinated three times with DCs loaded with CT-E7 and intratumoural CpG. Our vaccination protocol led to complete eradication of 100% of the tumours, but we started the vaccinations at an earlier time-point. Another successful therapeutic vaccine consist of DCs loaded with a MHC class I-restricted E7 peptide fused to a sequence for intracellular delivery [238]. The KDEL sequence used enables the delivery of the peptide to endoplasmatic reticulum for efficient loading on MHC class I molecules and thus enhances antigen-presentation. One dose of this vaccine in mice with 10-day tumours induced tumour regression and long-time survival in 50% of the animals, but did not lead to complete tumour eradication. DNA vaccination is also a promising approach. Several therapeutic DNA vaccinations protocols have used chemotherapeutic agents as adjuvants [239-241]. Apigenin is one of the agents that has been used and it made the tumours more prone to lysis by CTLs and also enhanced apoptotic cell death [239]. Mice with 3 day-tumours that were vaccinated with a DNA vaccine encoding HPV16 E7 linked to a heat-shock protein combined with apigenin, had significantly reduced tumour growth and 60% of the animals were still alive 84 days post TC-1 challenge [239]. Taken together, these studies show that successful therapeutic vaccination most likely requires a combination of treatments.

IDO is a tryptophan-catabolizing enzyme which is expressed in different subsets of DCs. IDO can downregulate T-cell responses [44] and it is therefore suggested that it can negatively affect DC-based immunotherapies [46], possibly by attracting regulatory T cells [242]. CT-pulsing did not *per se* induce IDO transcription or IDO

enzyme activity in human DCs, which might be one important explanatory factor for the strong adjuvant property of CT. However, CT did prime DCs for CD40L-induced IDO transcription and enzymatic activity. The role of this priming capacity for DC efficacy *in vivo* remains to be determined.

Women who on repeated examinations have CIN-positive Pap-smears are signed up for surgery within 3 months after being diagnosed. Eight out of 35 patients in our study (23%) who underwent surgery had reversed to normal cytology at the time for operation, and six of these eight were not infected with any of the HPV types tested. This means that these women might have undergone surgery unnecessarily. It is obvious that the selection criteria for surgery is not optimal and that more reliable diagnostic tools need to be developed.

Around 291 million women in the world are estimated to be infected with HPV [83]. Despite the availability of screening programs in several countries approximately 450 000 of these women develop cervical cancer each year [83, 89]. The therapies that exist today for cervical cancer are surgery, chemotherapy and radiation therapy, and the treatment results for patients with recurrent disease are poor [147]. Cervical cancer still leads to around 230 000 deaths annually worldwide [90, 91], and thus there is a great need of additional therapeutic treatments. Most of the women who die of cervical cancer live in developing countries. Many of these women would probably never get access to a therapeutic DC vaccine against cervical cancer even if such a vaccine existed, considering that it is an expensive treatment requiring access to tissue culture facilities. In Sweden, around 450 women each year are diagnosed with cervical cancer and approximately 180 women die of this disease every year despite the organised screening programmes. The mean age for being diagnosed with cervical cancer is around 55 years, with 25% of the affected women being younger than forty years old. Considering the fact that the lives of so many young women could potentially be saved should warrant the cost to develop and use therapeutic vaccines against cervical cancer.

The combinational treatment of TLR ligands and DC vaccine could possibly be an effective treatment for HPV-induced malignancies as it both makes the tumour cells easier to target by effector cells and enhances the antigen-specific T-cell responses. The other advantage is that a DC vaccine could be tailor-made for each patient. The HPV E7 is uncomplicated to produce, and each patient could be given the appropriate E7 type based on e.g. HPV typing of cervical biopsies. However, we do not know if the combinational treatment of TLR ligands and DC vaccination would have any impact on metastatic disease since metastases could be difficult to track and reach with local TLR ligand treatments.

CONCLUDING REMARKS

In this thesis I have developed and evaluated a potential therapeutic immunotherapy against HPV-induced malignancy. The immunotherapy consists of two components; dendritic cells that have been exposed *in vitro* to the HPV oncogene E7 conjugated to cholera toxin (CT-E7-DCs), and unmethylated CpG-rich oligonucleotides (CpG). Both these components could by themselves reduce tumour growth *in vivo*, but a successful treatment was only achieved when both compounds were used simultaneously. Thus, intravenous injection of CT-E7-DCs together with intra-tumoural administration of CpG led to a complete eradication of an HPV16 E7-expressing experimental tumour in mice. The CT-E7-DCs activated cytotoxic T-cells, which were fundamental for tumour eradication, whereas CpG enhanced the expression of MHC class I and II molecules, induced the production of chemokines (MIP-1 α , RANTES and IP-10), and led to an influx of CD11-positive DCs. I also evaluated whether the concept of DC vaccination, using CT-conjugated HPV16 E7 as antigen, would be feasible in a human setting. CT-E7-DCs induced comparably strong T-cell recall responses (compared to E7-pulsed DCs) in cells from women with different grades of HPV-induced disease, and these responses were characterised by elevated levels of Th1 cytokines. Interestingly, T cells from women who were not infected with HPV16 at the time of analysis responded equally well to CT-HPV16 E7-DCs as T cells from women with an ongoing HPV16 infection, implying that the CT-HPV16E7-DCs might confer protection also to HPV types other than HPV16. The enhanced T-cell activating potential of CT-treated human DCs was associated with a lack of expression of the immunomodulatory enzyme IDO, which has a strong immunosuppressive function on T-cell responses via its metabolism of tryptophan.

To conclude, our combinational anti-viral/anti-tumour therapeutic treatment consisting of intratumoural CpG injections and CT-E7-pulsed DC vaccination is efficient as therapeutic treatment of mice carrying E7-expressing TC-1 tumours. The CT-E7-DC vaccine also induced Th1 recall responses in cells from women who were or had been CIN positive. It is obvious that prophylactic vaccination and frequent screening are the most important ways to avoid infection and progression of premalignant transformations, respectively. Therapeutic vaccinations such as the DC vaccination presented in this thesis, could however be an addition to existing treatments of HPV-induced cervical disease in the future and, if successful, perhaps save life.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer i livmoderhalsen är en allvarlig cancerform. 450 000 kvinnor runtom i världen drabbas varje år, vilket leder till 230 000 kvinnors död. Livmoderhalscancer orsakas av ett virus som heter humant papillomavirus (HPV), som överförs via sexuella kontakter. HPV är en vanlig virusinfektion. Man räknar med att 75 procent av alla sexuellt aktiva individer någon gång i livet drabbas av HPV-infektion. Hos de flesta läker infektionen ut av sig själv, men en ihållande infektion kan leda till cellförändringar i livmoderhalsen och i värsta fall livmoderhalscancer. I Sverige kallas kvinnor regelbundet till undersökningar där man tar ett cellprov på livmoderhalsen och letar efter cellförändringar. Man kan då skära bort det infekterade området. Trots detta insjuknar i Sverige varje år ungefär 450 kvinnor i livmoderhalscancer. Många av dem är unga och medelåldern i Sverige för dem som drabbas är 55 år. En fjärdedel är under 40 år. Behandlingen för denna sjukdom är kirurgi, cellgifter och strålning. Om canceren har spridit sig i kroppen är den svår att bota och ungefär 180 kvinnor i Sverige dör varje år i livmoderhalscancer. De vacciner mot HPV som finns tillgängliga idag skyddar bara individer som inte redan har smittats. Syftet med arbetet som ligger till grund för denna avhandling var att försöka utveckla en behandling för HPV-infektion och livmoderhalscancer.

Som strategi för att utveckla en behandling har jag valt att försöka stimulera kroppens eget immunförsvar för att känna igen virusinfekterade celler eller tumörceller. I mitt avhandlingsarbete har jag därför utvecklat en kombinationsbehandling i möss som gör att djurens eget immunförsvar förmås att döda av virusinfekterade celler och tumörvävnad. Behandlingen består av två komponenter. Den ena komponenten gör att immunsystemet lär sig att känna igen en tumörcell. Den andra komponenten synliggör tumörcellen för immunsystemet. Med denna behandling kan tumörsjuka möss bli helt friska. Jag har inte testat behandlingen på människa, men jag kan i provrörsförsök visa att behandlingen har en liknande effekt på våra celler.

Det är uppenbart att vaccin och regelbundna cellprovskontroller är viktigast för att undvika smitta med HPV och för att upptäcka cellförändringar i tid. Men utvecklingen av nya behandlingar (som till exempel den kombinationsbehandling som beskrivs i min avhandling) kan vara värdefulla och kan kanske i framtiden, om de lyckas, rädda fler liv.

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